IDENTIFICATION OF BACTERIAL Fc RECEPTORS
AND CHARACTERIZATION OF A GROUP C STREPTOCOCCAL Fc RECEPTOR

By

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KEY TO ABBREVIATIONS

EA   Erythrocytes sensitized with subagglutinating doses of
            antibody

EDTA-gel   0.15 M Veronal buffered saline, pH 7.4, containing 0.01 M
            trisodium ethylenediamine-tetraacetate and 0.1% gelatin

FcR   Fc receptor contained on or obtained from any bacteria

FcRc   Fc receptor contained or obtained from the group C
            streptococcal strain 26RP66

I.M.   Intramuscularly

PA   Protein A, the Staphylococcus aureus Fc receptor

PBS   0.15 M Phosphate buffered saline, pH 7.4

SRBC   Sheep red blood cells

VBS-gel   0.15 M Veronal buffered saline, pH 7.4, containing 0.001 M
            Mg^{2+} and 0.00015 M Ca^{2+}, and 0.1% gelatin

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December 1983

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Major Department: Immunology and Medical Microbiology

The ability of bacteria to remove IgG in the presence of an

equimolar amount of $F(ab')_2$ fragments was used to identify

streptococci with Fc receptors on their surface. A quantitative

competitive binding assay was developed for the measurement of soluble

Fc receptors during extraction and purification procedures. An

antibody prepared against protein A, the staphylococcal Fc receptor,

was used to demonstrate that selected streptococcal Fc receptors and

protein A were antigenically distinct. Using these assays, a group C

streptococcus with high levels of Fc receptor activity on its surface

was selected for further study. The Fc receptor was extracted in high

yield by lysis of the bacteria following infection with bacteriophage

and purified by sequential chromatography on cellulose phosphate, DEAE

and selective elution from a column of immobilized human IgG. Four

hundred micrograms of the purified protein was obtained per gram (wet

weight) of bacteria extracted. The affinity purified receptor was

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functionally homogeneous in binding to the Fc region of human IgG, however, 4 major protein bands were observed on both nondenaturing and SDS polyacrylamide gels. Antibody prepared against the major 64,000 dalton protein was capable of reacting with all the fractions and competitive binding studies suggested that the purified Fc receptor is a single receptor and that the differences in charge and size were due to covalently bound cell wall constituents. Comparison of the group C streptococcal Fc receptor (FcRc) and protein A indicated that although they can compete with each other for binding to the Fc region of human IgG, the receptors are antigenically unrelated. Differences in species and subclasses reactivity showed that FcRc bound more efficiently to goat, sheep, and cow IgG, while protein A bound more efficiently to dog IgG. Differences were also observed in the reactivity of the two bacterial Fc receptors towards human IgG suclasses. The reactivity of the soluble FcRc corresponds to a type III streptococcal Fc receptor classified by the reactivity of intact bacteria. This is the first report of the isolation in high yield of a functionally homogeneous type III receptor.

CHAPTER ONE
INTRODUCTION

Bacterial receptors capable of reacting with the Fc region of various classes and subclasses of immunoglobulins from mammals have been reported on a number of strains of staphylococci (Jensen, 1958; Forsgren and Sjöquist, 1966) and streptococci (Kronvall, 1973). The most extensively studied of these is the Fc receptor isolated from Staphylococcus aureus and designated protein A.

Staphylococcal Protein A

Distribution, Isolation and Properties

Protein A is produced by most Staphylococcus aureus strains as a cell surface and/or secreted product. Studies of the distribution of protein A among staphylococci indicate it is present on the surface of, or secreted by, approximately 90% of all staphylococci studied (Langone, 1982a; Sperber, 1976). There are, however, marked quantitative differences between the levels of protein A on different staphylococci. The most widely studied Staphylococcus aureus, Cowan I strain, has large quantities of surface protein A while the Staphylococcus aureus Wood strain expresses very low levels (Freimer et al., 1979). Initial attempts to extract protein A by heating or using lysozyme resulted in a heterogeneous product (Forsgren, 1969; Forsgren and Sjöquist, 1969). A more homogeneous form of protein A has been described following treatment of Staphylococcus aureus Cowan strain

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with lysostaphin. This receptor has been shown to be an elongated 42,000 dalton protein (Sjöquist et al., 1972; Björk et al., 1972), which binds selectively to the Fc region of certain immunoglobulin species, classes and subclasses (Kronvall et al., 1970; Richman et al., 1982). A number of biological properties have also been attributed to protein A. Soluble and insoluble forms of protein A stimulate proliferation of human B cells in in vitro culture systems (Romagnani et al., 1981) and complexes of protein A and IgG are capable of activating the classical complement pathway (Ståhlenheim et al., 1973). Additionally, injection of protein A into guinea pigs is capable of causing Arthus and anaphylactoid type hypersensitivity reactions (Gustafson et al., 1968).

Immunochemical Applications

The ability of protein A to react selectively with the Fc portion of immunoglobulin molecules has been utilized in a variety of immunologic procedures primarily for purifying and quantifying reactive classes and subclasses of IgG (for review see Goding, 1978; Langone, 1982a). Protein A has also been reported to bind to certain species of IgA (Patrick et al., 1977) and IgM (Lind et al., 1975). These properties have been useful in establishing the existance of different subclasses within isotypes, enabling them to be isolated and characterized with ease (Lind et al., 1975; Patrick et al., 1977). Protein A can be efficiently used to remove IgG from human serum for use in a variety of assays to detect non-IgG antibodies (Langone et al., 1979; Boyle and Langone, 1979) and protein A-bearing staphylococci have been used extensively in place of second or precipitating antibody in a variety of radioimmunoassays (Goding, 1978). More recently

radiolabeled or enzyme-linked protein A has been used as a universal tracer in a number of assays for cell surface and soluble antigens (Langone 1978, 1980b; Langone et al., 1979a,b; Boyle and Langone, 1979; Gee and Langone, 1981, for recent review see Langone 1982b). The usefulness of protein A for immunochemical studies is only limited by the range of species, isotypes, and subclasses with which it reacts.

Streptococcal Immunoglobulin Fc Receptors (FcR)
Distribution of Fc Receptors Among Streptococci

In 1973, Kronvall (1973) found that groups A, C and G streptococcal strains were capable of agglutinating red blood cells sensitized with subagglutinating doses of antibody (EA), indicating the presence of an IgG Fc receptor on the surface of these bacteria. The distribution of immunoglobulin G-Fc receptors among various streptococcal strains has been studied by a variety of methods. Methods for detection of IgG FcR on the surface of streptococci include: 1) agglutination of EA (Kronvall, 1973; Christensen and Kronvall, 1974), 2) binding of radiolabeled polyclonal or myeloma IgG (Christensen and Oxelius, 1974; Myhre and Kronvall, 1977, 1980a; Myhre et al., 1979; Ericson et al., 1979) and 3) mixed reverse passive antiglobulin haemagglutination assay (Freimer et al., 1979). In this test bacterial Fc receptors are detected by preincubating bacteria with purified Fc fragments of IgG. These bound fragments are subsequently detected by agglutination of red blood cells that have antibodies to Fc regions of IgG coupled to their surface.

The results from these investigations are summarized in Table 1-1, and suggest that Fc receptors can be found with a high frequency on

TABLE 1-1

Distribution of IgG Fc Receptors on Streptococci

Source	Lancefield Group	Species	No. FcR Positive / No. Tested		Method of Detection	Reference
Human	A	S. pyogenes	9/32	(28%)	c	Kronvall
	C	ND	5/10	(50%)		(1973)
	G	--	1/15	(6%)		
Human	A	S. pyogenes	3/3	(100%)	a,b	Christensen
	B	S. agalactiae	1/3	(33%)		and Oxelius
	C	ND	3/3	(100%)		(1974)
	G	--	2/3	(63%)		
Human	A	S. pyogenes	19/30	(63%)	a	Myhre and
	B	S. agalactiae	0/40	(0%)		Kronvall
	C	ND	25/30	(83%)		(1977)
	D	ND	0/30	(0%)		
	G	--	25/30	(83%)		
Human	A	S. pyogenes	4/7	(57%)	d	Freimer
	B	S. agalactiae	0/2	(0%)		et al.,
	C	ND	2/2	(100%)		(1979)
	D	ND	0/3	(0%)		
	G	--	2/2	(100%)		
Human	C	S. equisimilis	10/10	(100%)	b	Myhre and
Human	C	S. dysgalactiae	12/12	(100%)		Kronvall
Human & Non-Human	C	S. zooepidemicus	18/18	(100%)		(1980a)
Non-Human	C	S. equii	7/7	(100%)		
Human	G	--	17/20	(85%)	a	Myhre et al.
Bovine (β-hemolytic)	G	--	13/16	(81%)		(1979)
Bovine (α-hemolytic)	G	--	0/18	(0%)		
Human	NG	S. mutans	0/11	(0%)	a,b	Ericson
Oral	NG	S. sanguis	0/6	(0%)		et al.
Isolates	NG	S. milior	0/5	(0%)		(1979)
	NG	S. salivarius	0/5	(0%)		
	NG	S. milleri	0/4	(0%		

a = uptake of radiolabeled polyclonal IgG
b = uptake of radiolabeled human myeloma IgG
c = agglutination of sheep red cells (SRBC)
d = mixed reverse passive hemagglutination

ND = Not Determined
NG = Not Grouped

human isolates of group A (35/72, 49%), group C (45/55, 82%) and group G (47/70, 67%) streptococci. Although no direct correlation between M proteins found on group A strains and FcR has been found, it has been noted that some strains are more likely to produce Fc receptors than others, e.g., serotype M8 (5 of 6 strains tested) and serotype M22 (23 of 23 strains tested) were positive for either surface FcR or hot-acid extractable FcR, while none of the 54 serotype M12 strains had either surface or hot-acid extractable FcR (Havlíček, 1978; Burova et al., 1981). Recent reports indicate that IgG Fc receptors can be detected in hot-acid extracts of serotype M12 positive Fc receptor negative strains following passage in mice. Appearance of an extractable FcR, is accompanied by a loss of the surface M type 12 antigen (Burova et al., 1980, 1981; Christensen et al., 1979a).

The stability of FcR production by a given bacterial strain during subculture or storage is an important consideration when selecting strains for study of these receptors. There are conflicting reports concerning the stability of Fc receptors on the bacterial cell surface. Christensen and Kronvall (1974) reported that repeated subculturing of groups A, B, C, D, and G streptococci did not suppress the ability of these bacteria to agglutinate EA. Havlíček (1978) looked at Fc receptor activity in hot-acid extracts of a number of fresh isolates, collection strains and freeze-dried strains of group A streptococci and reported that Fc receptors are found more frequently on fresh than on collection or freeze-dried strains. Extracts of 38 of 175 (22%) collection strains and 10 of 32 (31%) freeze-dried strains had Fc receptors while 49 or 49 (100%) fresh isolates had detectable levels of Fc receptor activity as measured by the ability to agglutinate EA.

Havlíček does not indicate whether these fresh isolates were followed, after subculturing or storage, for loss of FcR activity. Schalén et al. (1983) found that 10 of 20 (50%) reference strains of group A streptococci of various M types had Fc receptors as detected by binding of radiolabeled polyclonal and monoclonal IgG. Additionally, fresh isolates of group A streptococci have been reported to have a greater capacity to adsorb radiolabeled myeloma IgA than do collection strains (Christensen and Oxelius, 1975; Schalén, 1980). Although the frequency of FcR positive group A strains appears to be higher in fresh isolates than in older cultures, the stability of Fc receptors appears to follow no precise pattern during subculturing and storage.

Immunoglobulin Species and Subclass Reactivities

Characterization of the Fc-reactivity of streptococci is based on the ability of serum or IgG fractions from different animal species to inhibit the binding of radiolabeled human IgG to a variety of bacteria (Myhre and Kronvall, 1977, 1980a; Myhre et al., 1979). Inhibition experiments with isolated Fc fragments demonstrated that the binding site for the immunoglobulin Fc receptor is located in the CH_2 domain of the Fc fragment of the immunoglobulin molecule (Christensen et al., 1976; Myhre and Kronvall, 1980b). Subclass reactivity has been measured by the ability of a variety of radiolabeled myeloma proteins to bind directly to the bacteria (Kronvall et al., 1979a; Myhre and Kronvall, 1981b). Using these assays Myhre and Kronvall have described five different Fc receptor types (see Tables 1-2 and 1-3).

These results are semi-quantitative and only reflect major differences in reactivities. Similarily, known differences in affinities of both staphylococcal protein A (Langone, 1978) and streptococcal

TABLE 1-2

Species and Subclass IgG Reactivities of Bacterial Fc Receptors[a]

		IgG Fc Receptor Type[b]				
		I	II	III	IV	V
Human	IgG_1	+++	+++	+++	+	+++
	IgG_2	+++	+++	+++	−	+++
	IgG_3	−	+++	+++	−	−
	IgG_4	+++	+++	+++	+	+++
	IgA^c	−	−	−	−	−
	IgM	−	−	−	−	−
Mouse	IgG_1	+	−	+	NT^d	NT
	IgG_{2a}	+++	−	+++	NT	NT
	IgG_{2b}	+++	−	+++	NT	NT
	IgG_3	+++	−	+++	NT	NT
Cow	IgG_1	−	−	+++	−	−
	IgG_2	+++	−	+++	−	+
Sheep	IgG_1	−	−	+++	−	+
	IgG_2	+++	−	+++	−	+
Goat	IgG_1	+	−	+++	−	+
	IgG_2	+++	−	+++	−	+
Horse	IgG(ab)	+	−	+++	−	+
	IgG(c)	+	−	+++	+	+
	IgG(T)	−	(+)	(+)	(+)	(+)
Rabbit	IgG	+++	+++	+++	NT	+++
Guinea Pig	IgG	+++	−	+++	NT	NT
Rat	IgG	−	−	−	NT	−
Dog	IgG	+++	−	−	NT	−
Cat	IgG	+++	−	−	NT	−
Pig	IgG	+++	+++	+++	NT	+++
Chicken	IgG	−	−	−	NT	NT

+++ = indicates strong reactivity   + = indicates low reactivity
(+) = weak, atypical reactivity
  a = Summarized from Kronvall, 1973; Myhre and Kronvall, 1977, 1980a,b, 1981b
  b = see TABLE 1-3
  c = IgA Fc receptor is found on certain group A streptococci, but is
     distinct from the IgG Fc receptor (Christensen and Oxelius,
     1975; Kronvall et al., 1979a; Schalén et al., 1980).
  d = NT, not tested

TABLE 1-3

Types of Bacterial Fc Receptors[a]

Type of IgG Fc Receptor	Bacterial Species
Type I	Staphylococcus aureus (Protein A)
Type II	Group A streptococci
Type III	Strep. equisimilis (group C)[b] Strep. dysgalactiae (group C) Human group C streptococci
Type IV	Bovine β-hemolytic group G streptococci
Type V	Strep. zooepidemicus (group C)

a = From Myhre and Kronvall, 1981b.
b = The group C strain Streptococcus equii binds negligible levels of human IgG (Myhre and Kronvall, 1980a).

Fc receptors are not reflected in the results presented in Tables 1-2
and 1-3 (Kronvall, 1973; Myhre and Kronvall, 1977, 1980b, 1981a). More
sensitive assays using purified protein A have been very useful in
defining its relative species and subclass reactivity. For example,
using a quantitative competitive binding assay Langone (1978) has shown
that 50% inhibition of binding of ^{125}I-PA to immobilized rabbit IgG
required 60 ng of rabbit, human or guinea pig IgG, 135 ng of pig IgG,
4,500 ng of mouse IgG and 40,000 ng of sheep IgG. Similar assays with
streptococcal Fc receptors have not been carried out since purified
receptors and hence the radiolabeled tracers are not available.

Pathogenicity

No one factor has been shown to be solely responsible for the
pathogenicity of streptococci, but factors such as M proteins (Bisno,
1979; Lancefield, 1954; Todd and Lancefield, 1928), opacity factor
(Maxted and Widdowson, 1972), hyaluronic acid (Kass and Seastone,
1944), neuraminidase (Davis et al., 1979) and the M-associated proteins
(Maxted and Widdowson, 1972) have all been implicated. There is little
information available on the relationship of streptococcal Fc receptors
and pathogenicity.

Passage of streptococci in mice has been used to increase mouse
virulence and passage in fresh human blood has been used to select
phagocytic resistant strains, both of these methods are currently in
use for the study of virulence factors. Several studies of FcR produc-
tion and virulence have suggested a possible role for Fc receptors in
pathogenesis. Mouse passage of a number of group A streptococci (M
serotypes 3, 12 and 46) has resulted in increased virulence in mice,
loss of M protein and a concurrent production of an extractable Fc

receptor (Christensen et al., 1979a; Burova et al., 1980). Burova et al. (1981) extracted and semipurified an Fc receptor from a group A serotype M15 streptococcus that was highly virulent in mice. When this receptor was mixed with a serotype M12 strain of low virulence, an increase in virulence was observed both in vivo and in vitro.

There has been recent evidence to suggest that the synthesis of at least two pathogenic factors of group A streptococci, M protein and opacity factor is under extrachromosomal regulation (Cleary et al., 1975; Spanier and Cleary, 1980). Burova et al. (1983) have looked at the role of plasmids in the expression of antiphagocytic activity, opacity factor, IgG, and IgA receptors and suggest that expression of these factors may be triggered by insertion of plasmid DNA into the bacterial chromosome.

These findings show that Fc receptor activity can be induced, or expression enhanced, along with other factors that have been associated with virulence. Before the importance of Fc receptors in virulence can be critically assessed, purified Fc receptors will have to be isolated and studied in more detail.

Isolation and Properties

The earliest attempts to extract a soluble streptococcal Fc receptor employed the hot-acid or Lancefield technique (Lancefield, 1928). This method involves adjusting the pH of a bacterial suspension to pH 2.0, heating in a boiling water bath for 10 mins and neutralizing the pH. A modification of this procedure, the hot-alkaline extraction, is carried out in the same manner except that the pH is adjusted to 10.0. These methods are commonly used for the extraction of the streptococcal group specific carbohydrate and streptococcal proteins,

including the M protein from group A streptococci. Extracts obtained
by these methods from groups A, C, and G streptococci were able to
agglutinate antibody sensitized red blood cells (Christensen and
Kronvall, 1974; Christensen and Holm, 1976; Havlíček, 1978; Schalén et
al., 1978, 1980; Christensen et al., 1979a,b), inhibit the binding of
radiolabeled IgG to intact streptococci (Christensen and Oxelius, 1974;
Christensen and Holm, 1976) and precipitate human serum or IgG
components (Schalén et al., 1978, 1980; Christensen et al., 1979b;
Grubb et al., 1982). Extraction of a group C streptococcus by phage
lysis, and a group A strain by autoclaving at 120°C for 30 minutes, was
used by Christensen and Holm (1976) to obtain soluble Fc receptors.
The same investigators reported only limited success when hot-acid or
hot-alkaline methods, ultrasonic treatment, X-pressing or Mickle
disintegration was used on a limited number of strains (see Table 1-4).
It is difficult to compare extraction methods, purification yields, or
molecular weight determinations of the products obtained since four
different strains were extracted, each by a different method.
Additionally, each investigator used a different assay to quantitate
the solubilized Fc receptors and for determining the molecular weight.
Only Grubb et al. (1982) using a group A (serotype M15) streptococcal
strain was able to obtain a functionally homogeneous product. The Fc
receptor was solubilized by hot-alkaline extraction and purified by
DEAE ion exchange chromatography and immunoadsorption on an IgG column
and subsequent elution with 0.1 M sodium acetate, pH 3.5, containing
0.5 M NaCl. All steps in the extraction and purification of this
receptor required the presence of protease inhibitors. The authors
report that when protease inhibitors were not included during the

extraction and isolation, results were not reproducible and Fc receptor activity often decayed rapidly. Six hundred μg of purified Fc receptor could be obtained from 60 g bacteria (wet weight), and a yield of 11% Fc receptor present in the crude hot-alkaline extract was achieved. The authors also report that some charge and size heterogeneity on SDS-polyacrylamide and agarose gel electrophoresis was evident in the purified material, even though it was determined to be functionally pure based on its ability to bind immobilized monoclonal or polyclonal IgG.

The Fc receptor(s) of group A streptococci have been shown to be distinct from the M proteins (Havlíček, 1978; Christensen et al., 1979b; Schalén et al., 1980), the group carbohydrate and peptidoglycan (Christensen et al., 1979b; Schalén et al., 1980) as well as lipoteichoic acid (Schalén et al., 1980). A group C Fc receptor extracted by phage lysis and a group A Fc receptor extracted by heating to 120°C, were both shown to be sensitive to trypsin and heat treatment at 95°C for 10 mins at pH 2.0 (Christensen and Holm, 1976). The group A streptococcal Fc receptor extracted and purified by Grubb et al. (1982) (see Table 1-4) was found to have a molecular weight of 29,500 when determined by SDS-polyacrylamide gel electrophoresis. However, gel chromatography run under nondenaturing conditions revealed a hydrodynamic volume between that of IgG and IgA, indicating that the native molecule is either elongated or forms oligomers. Grubb et al. (1982) have also determined the amino acid composition of their group A streptococcal Fc receptor and shown that it is distinct from that of staphylococcal protein A.

TABLE 1-4

Characteristics of Fc Receptors Isolated from Streptococci

Strain	Method of Extraction	Method of Detection of Soluble FcR Activity	Purification	Molecular Weight (daltons)	Comments	Reference
Group C (S. azgazardah)	Phage Lysis	a	1) Immobilized IgG-eluted with 3 M KSCN 2) Gel filtration on Sephadex G-75	60,000 (by gel filtration)	Partial purification obtained	Christensen and Holm (1976)
Group A (Type M56)	120°C, 30 mins	b	Gel filtration on Sephadex G-75	60,000 (by gel filtration)	Partial purification obtained	Christensen and Holm (1976)
Group A (Type 8)	Hot-Acid	b	Gel filtration on Sephadex G-200	100,000 (by gel filtration)	Partial purification obtained	Havlíček (1978)
Group A (Type 15)	Hot-Alkaline	c	1) DEAE ion exchange chromatography 2) Immobilized IgG-eluted at pH 3.5	29,500 (by SDS-PAGE)	Purified to functional homogeneity. Protease inhibitors required during purification	Grubb et al. (1982)

a = inhibition of binding of radiolabeled IgG to intact Fc receptor positive streptococci

b = agglutination of antibody sensitized red blood cells

c = immunoprecipitation in agarose gels containing human myeloma IgG, and 10% (w/v) Dextran T10

The role of Fc receptors in the pathogenesis of streptococcal infections and their potential as immunochemical reagents has not been fully assessed. Before these types of studies can be undertaken, methods of obtaining reasonable quantities of purified Fc receptors must be developed.

Summary

The existence of immunoglobulin Fc receptors on streptococci was established by Kronvall in 1973. Inhibition of binding of radiolabeled IgG to streptococci by various species, classes and subclasses of immunoglobulins has been used to determine the specificity of immunoglobulin reactivity of a variety of streptococcal strains. Based on the pattern of binding of differing species of immunoglobulins to streptococci, four types of streptococcal Fc receptors have been described that are distinct from the staphylococcal protein A receptor.

Soluble streptococcal Fc receptors have not been as extensively studied as staphylococcal protein A. Attempts to extract and purify streptococcal Fc receptors using phage lysis, heat and hot-acid or hot-alkaline extraction procedures have met with limited success. The use of protease inhibitors during the extraction procedure has recently been reported to give increased yields of a stable product extracted by the hot-alkaline method. The extracted material is capable of agglutinating antibody sensitized red blood cells, inhibiting the uptake of radiolabeled IgG by intact streptococci and precipitating human IgG in agarose gels containing dextran. The molecular weights of the streptococcal Fc receptors extracted to date have a range from

30,000 to 100,000 daltons, depending on the organism extracted, the method of extraction and the method used for molecular weight determinations, i.e., gel filtration or SDS polyacrylamide gel electrophoresis.

The biological properties of streptococcal Fc receptors have not been extensively characterized. Streptococcal Fc receptors are clearly distinct from the streptococcal virulence factor, the M proteins found on group A streptococci, but may be associated with them. Recent reports indicate that production of streptococcal Fc receptors may be induced or enhanced by mouse passage of selected bacterial strains, or by the insertion of plasmid DNA into the bacterial chromosome. Additionally, partially purified Fc receptors have been shown to alter pathogenicity and phagocytosis of selected group A strains in in vivo and in vitro experiments.

The sparsity of information on the physicochemical and biological properties of streptococcal Fc receptors is due, in large part, to the absence of reproducible sensitive assays to detect these proteins. A number of problems have been encountered with the methods currently in use for the detection and quantitation of streptococcal Fc receptors. Hemagglutination reactions are frequently weak and best results are achieved when human red cells sensitized with the Ripley antibody, a human anti-Rh antibody, are used. Hemolysis of red blood cells by bacterial hemolysins has been reported, but may be overcome by heat treatment of culture supernatants or extracts prior to testing. When using ^{125}I labeled IgG to detect Fc receptors, high levels of adherence of ^{125}I IgG to reaction vessels and entrapment by bacteria have been observed, despite the presence of nonspecific

protein sources and low levels of detergent. Self-aggregating streptococci can cause entrapment of unsensitized, as well as sensitized red blood cells, and entrapment of ^{125}I-labeled IgG making interpretation of results difficult.

The ability to identify the bacterial groups responsible for selective reaction with the Fc region of IgG and to isolate them in high yields in a form that maintains functional activity would be clearly useful. In particular, bacterial groups which react with a wider range of immunoglobulin species, classes, and subclasses than protein A would enable the immunochemical techniques currently employing protein A to be expanded. For example, some group A, C and G streptococci have been reported to react with human IgG$_3$ and group C streptococci have been found that react with sheep and cow IgG. These activities are not associated with protein A. Isolation of bacterial groups with fewer species or subclass reactivities than protein A would enable techniques to be developed using both bacterial reactivities to focus on a narrower range of specificities. The usefulness of bacterial Fc receptors in immunochemical studies is at present limited only by the range of species, class and subclass reactivities with which these receptors will react. The ability to isolate Fc receptors in a functionally homogenous, soluble form with reactivities different from those of protein A would allow the highly successful approaches using staphylococcal protein A to be extended. Additionally, purified bacterial Fc receptors from different types of streptococci will be required to study IgG-FcR interactions and to define their role in vivo.

Consequently, the purpose of this study was to identify streptococci with surface receptors capable of reacting selectively with the Fc region of immunoglobulin molecules. Once methods for the detection of such molecules were developed, suitable Fc receptor-positive streptococcal strains were used to develop methods for the extraction and purification of these receptors. Emphasis was focused on streptococcal Fc receptors with IgG isotype species or subclass reactivities different from those of staphylococcal protein A. Once Fc receptors were isolated, their physicochemical and biological properties were characterized and their usefulness in a variety of immunoassays were evaluated. The specific aims of this study were to:

(1) Develop methods for screening bacteria for Fc receptors and for quantitating these receptors in a soluble form (Chapter 2).

(2) Determine if the streptococcal Fc receptors were distinct from staphylococcal protein A (Chapter 3).

(3) Isolate and physicochemically characterize one of these Fc receptor(s) (Chapter 4).

(4) Characterize the functional and biological activities of the isolated Fc receptor(s), e.g., species immunoglobulin reactivities (Chapter 5).

CHAPTER TWO
METHODS FOR SCREENING BACTERIA FOR Fc RECEPTORS
AND FOR QUANTITATING THESE RECEPTORS IN SOLUBLE FORM

Introduction

The initial aim of this study was to develop methods for detecting

cell surface and soluble Fc receptors. Currently, two basic types of

assays have been used to measure Fc receptors on or secreted by

streptococci. The first utilizes hemagglutination which measures the

ability of intact bacteria or soluble extracts to cause agglutination

of red cells sensitized with subagglutinating concentrations of anti-

red cell antibodies (Kronvall, 1973; Christensen and Kronvall, 1974;

Havlíček, 1978). The second assay measures the ability of bacteria to

bind ^{125}I labeled IgG (Christensen and Oxelius, 1974; Christensen

et al., 1976; Myhre and Kronvall, 1977).

Both of these assays suffer from a number of technical limita-

tions. For example, a large number of strains of streptococci self

associate and cause apparent agglutination of unsensitized red cells

(Christensen and Kronvall, 1974; Freimer et al., 1979). In studies

utilizing ^{125}I labeled IgG high background levels of adherence of

label to the reaction vessel have made it difficult to measure

accurately Fc-reactivity (Christensen and Oxelius, 1974; Myhre and

Kronvall, 1977).

This chapter describes a method for the selective, semiquantita-

tive measurement of Fc receptors on the streptococcal cell surface.

The method described is rapid, reproducible, not subject to high
background values and can be used with auto-agglutinating bacteria.
The second part of this chapter describes the development of a competi-
tive binding assay for the quantitation of soluble Fc receptors.

Materials and Methods

Bacteria Strains, Media and Growth Conditions

Laboratory strains and fresh isolates of β-hemolytic streptococci
and Staphylocococcus aureus strains were used in these assays. All
strains were grown in Todd-Hewitt broth (Difco) for 18-24 hrs at 37°C.
They were heat killed at 80°C for 5 mins (Kronvall et al., 1979b),
harvested by centrifugation and washed twice in phosphate buffered
saline (PBS), pH 7.2, containing 0.02% Na azide. The optical density
at 550 nm was determined to standardize the concentration of organisms
used in subsequent tests. Sodium azide was added to a final
concentration of 0.02% to culture supernatants which were stored at 4°C
until testing.

Staphylococcus aureus Cowan I served as a protein A (PA)
producing positive control.

The β-hemolytic streptococci were grouped by the Phadebact
Streptococcus Test, Pharmacia Diagnostics.

Immunoglobulin

Stock human and rabbit IgG were prepared by chromatography of
normal human or rabbit serum on DEAE cellulose (Boyle and Langone,
1980). Aliquots were stored at -70°C until use.

Purified Protein A

Purified PA was obtained from Pharmacia Fine Chemicals,
Piscataway, New Jersey.

Iodination of PA and IgG

Purified PA (Pharmacia) was radioiodinated by the mild lactoperoxidase method using enzyme beads (Bio-Rad) (Morrison et al., 1971). The labeled protein was separated from free iodine by passage over a G25 column (PL-10, Pharmacia) and collected in veronal buffered saline, pH 7.4 containing 0.001 M Mg^{2+} and 0.00015 M Ca^{2+}, and 0.1% gelatin (VBS-gel). The labeled material routinely had a specific activity of approximately 0.3 mCi/mg.

$F(ab')_2$ Preparation

Human and rabbit IgG $F(ab')_2$ fragments were prepared by pepsin digestion of the stock IgG preparation by a modification of the method described by Nisonoff et al., (1960). Essentially, 20 mgs of IgG was incubated in 0.1 M Na acetate, 0.05 M NaCl pH 4.0 with 5% w/w of pepsin (Sigma) for 18 hrs at 37°C. The resulting $F(ab')_2$ fpagments were separated from undigested IgG and Fc fragments by chromatography on a Sephadex G-200 column in veronal buffered saline, pH 7.35. The peak corresponding to the $F(ab')_2$ fragments was pooled and any contaminating undigested IgG was removed by adsorption with PA-Sepharose (Pharmacia). The pooled PA adsorbed $F(ab')_2$ fragments were concentrated by negative pressure dialysis, aliquoted and stored at -70°C.

IgG Assay

Human IgG in solution was quantified by the procedure of Langone et al. (1977). In this assay, 0.2 mls of a test sample or buffer was mixed with 0.1 ml of a standard suspension of rabbit IgG covalently coupled to agarose beads (Immunobead R-1 Bio Rad Laboratories) and 0.1 ml of ^{125}I PA (approximately 20,000 cpm). After incubation at 37°C for 90 mins, 2 mls of veronal buffered saline containing 0.01 M

trisodium ethylenediaminetetraacetate and 0.1% gelatin (EDTA-gel) was added to each tube and centrifuged at 1,000 g for 5 mins and the supernatant fluid decanted. After an additional wash, the radioactivity associated with the beads was determined in a gamma counter (either Packard or LKB). The number of counts bound in the absence of fluid phase IgG was compared to the number of counts bound to the beads in the presence of known amounts of fluid phase IgG. The degree of inhibition was determined and a standard curve relating quantity of IgG to percent inhibition was generated. The quantity of IgG in the test sample can be determined by comparing the percent inhibition with the standard curve. Fifty percent inhibition of binding of ^{125}I-PA to the Immunobeads is consistently achieved with 30-50 ng of IgG.

Adsorption of IgG by Bacteria

The detection of Fc receptors on the surface of bacteria was determined by the ability of bacteria to adsorb IgG from solution. A standardized number of organisms, as indicated in the text, was added to 1 ml of VBS-gel containing 1 μg of IgG and incubated at ambient temperature for 1 hr. The bacteria were removed by centrifugation and the residual IgG in an aliquot of the supernatant fluid was measured as described above. Results are expressed as the percent of IgG adsorbed.

Protein A (PA) Assay

A two-stage assay for the detection of soluble PA or PA-like molecules is described in the text. This method is a modification of the competitive binding assay for the detection of soluble PA described by Langone et al., (1977).

Hemagglutination Assay

Soluble streptococcal and staphylococcal Fc-receptors were tested for their ability to agglutinate human red blood cells sensitized with subagglutinating doses of IgG (Sjöquist and Stålenheim, 1969; Kronvall, 1973; Winblad and Ericson, 1973). Human red cells were drawn in heparin, washed twice in EDTA-gel, once in VBS-gel, and resuspended to 3% in VBS-gel. Cells were sensitized to subagglutinating doses with commercial anti-Rh typing antiserum (Gamma Biologicals, Inc.) by incubating 0.2 mls of two-fold dilutions of anti-Rh antisera with 2 mls of 3% Rh positive red blood cells (RBCs) for 1 hr at 37°C. The cells were washed three times in VBS-gel to remove unbound antibody and resuspended to 0.3% in the same buffer.

The ability of bacterial culture supernatants to agglutinate sensitized erythrocytes was determined in V bottom micro-titer wells. Fifty μl of three-fold serial dilutions of streptococcal or staphylococcal culture supernatants was mixed with an equal volume of sensitized or unsensitized erythrocytes. Hemagglutination was scored following incubation at ambient temperature for 2 hrs and again after overnight incubation.

Lancefield Extraction

Bacteria for extraction were obtained after overnight growth at 37°C in Todd Hewitt broth. The bacteria were collected by centrifugation and washed once in PBS, pH 7.2. Lancefield extracts (Lancefield, 1928) were performed on approximately 0.1 g (wet weight) of bacteria suspended in 3 mls of 0.15 M NaCl. The pH was adjusted to 2.0 with 1 N HCl. The tubes containing the bacteria were placed in a boiling water bath for 10 mins, cooled on ice and the pH adjusted to 7.0 with 1 N

NaOH. The extracts were recovered after removal of bacteria by centrifugation and filtration through a 0.2 μm filter.

Lysostaphin Extraction

Lysostaphin extracts were performed according to the method of Sjöquist et al., 1972. Bacteria obtained as described above were extracted in 3 mls of 0.05 M Tris-HCl, 0.015 M NaCl, pH 7.5 containing 0.2 mgs lysostaphin (Sigma) and 10 μg DNase (Sigma). Following 4 hrs incubation at 37°C the extracts were recovered by centrifugation followed by filtration through a 0.2 μm filter.

Results

Quantitative Adsorption of Human IgG by Streptococci

The purpose of the experiments described in this chapter was to develop a rapid and selective assay to measure Fc receptors on streptococci, including autoagglutinating strains. A method was sought that would enable binding via the Fc region to be readily distinguished from binding through the $F(ab')_2$ region and nonspecific entrapment. The initial approach was to measure the ability of streptococci to adsorb IgG from solution.

In this procedure 1 ml of human IgG at a concentration of 1 μg/ml was added to a pellet containing approximately 10^{10} streptococci. The mixture was incubated for 1 hr at ambient temperature and the bacteria removed by centrifugation. The residual IgG in solution was measured in duplicate 0.2 ml aliquots of the supernatant using the competitive binding radio immunoassay described in the methods. This assay is based on the inhibition of ^{125}I protein A binding to immobilized IgG and is consequently a selective measure of IgG

Fc regions. Using this assay there was no detectable change in the concentration of IgG added to a blank tube and carried through the procedure.

The IgG adsorption assay was used to screen a number of fresh isolates of β-hemolytic streptococci. The results presented in Figure 2-1 indicate that different levels of IgG binding capacities could be detected by this method. The Cowan strain Staphylococcus aureus was included as a reference positive control and the Wood 46 strain as a reference low level positive control (Freimer et al., 1979).

In addition to the binding of the Fc region of IgG, streptococci may also bind IgG via the antigen combining sites. The human IgG used for adsorption had been isolated from a single donor and it would be unlikely that greater than 10% of the total immunoglobulin would be directed against bacterial antigens. However, to determine that the observed adsorption of IgG was Fc specific, the ability of the adsorbing streptococci (Figure 2-1) to remove IgG in the presence of an equimolar quantity of $F(ab')_2$ fragments was tested. The $F(ab')_2$ fragments were prepared from the same IgG source used in the adsorption assay and would contain the same distribution of IgG antigen reactivities. IgG $F(ab')_2$ fragments in the reaction mixture would compete with any binding of specific IgG antibody, resulting in decreased adsorption of the IgG. However, if binding was through the Fc region, no decrease would be observed when $F(ab')_2$ fragments were present. These studies could only be done because the radioimmunoassay for IgG I am using is based on the competitive binding of ^{125}I labeled

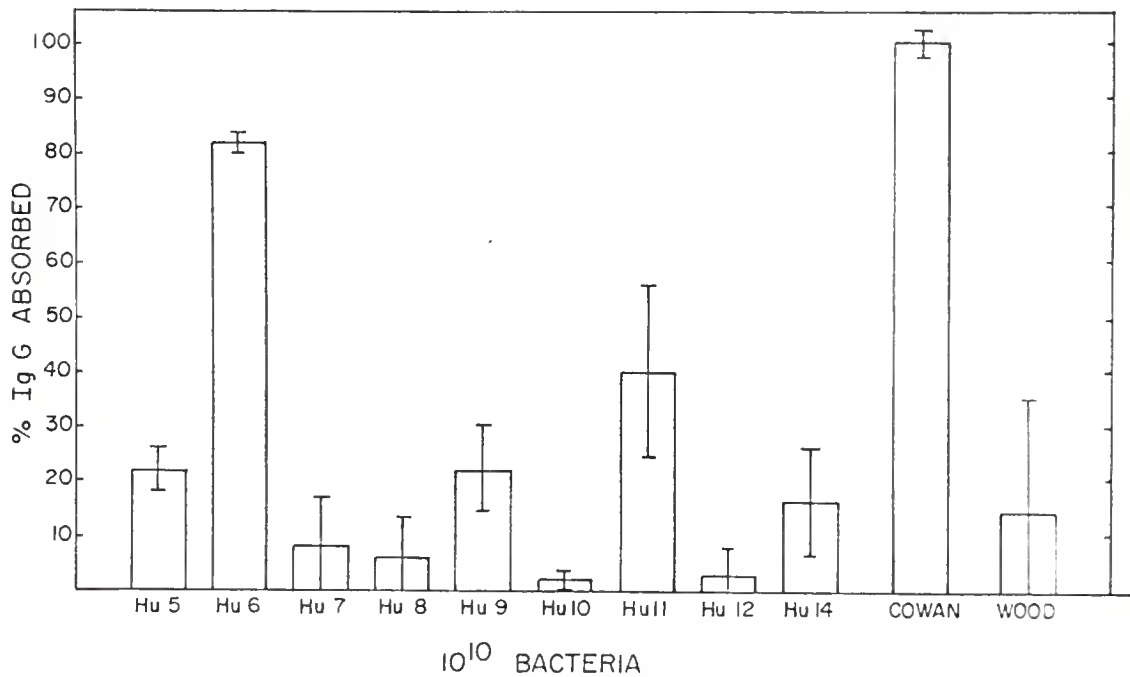Figure 2-1. Adsorption of human IgG by 1 x 10^10 streptococci. One μg of IgG was mixed with the number of bacteria indicated and the IgG remaining in an aliquot of the supernatant was determined. Results are expressed as the percent of IgG adsorbed by the bacteria, + the standard deviation. Hu6 is a group C streptococcus, the remainder belong to group A. Staph. aureus strains Cowan and Wood are shown for comparison.

protein A to the Fc region of immobilized IgG and F(ab')$_2$ fragments are not measured in this assay.

The results presented in Figure 2-2 indicate that the adsorbtion of IgG was not markedly altered when carried out in the presence of F(ab')$_2$ fragments. These results indicate that these group A and group C streptococci bind IgG through the Fc region of the immuno-globulin molecule. In all cases the capacity of individual strains to adsorb human IgG was related to the number of bacteria used in the assay. This is demonstrated in Figure 2-3 for two group A strepto-coccal strains. These results indicate that certain clinical isolates of streptococci have adsorbtive capacity for human IgG of approximately 5-35% of that of Staphylococcus aureus Cowan strain, under these assay conditions.

This approach can also be used to determine the species reactivity of bacterial Fc receptors by changing the source of IgG used for adsorption. A representative example of this approach, utilizing rabbit IgG and the corresponding F(ab')$_2$ fragments, is presented in Figure 2-4. A comparison of Figures 2-2, 2-3, and 2-4 demonstrates that the patterns of adsorption of human IgG and rabbit IgG were similar, with one exception. The group A strain, 529, adsorbed human IgG but failed to adsorb rabbit IgG. Functional differences between Fc-reactivity towards different species of IgG has previously been reported (Myhre and Kronvall, 1977). The assay being used to measure functional Fc receptors could be readily extended to other species of IgG classes or subclasses.
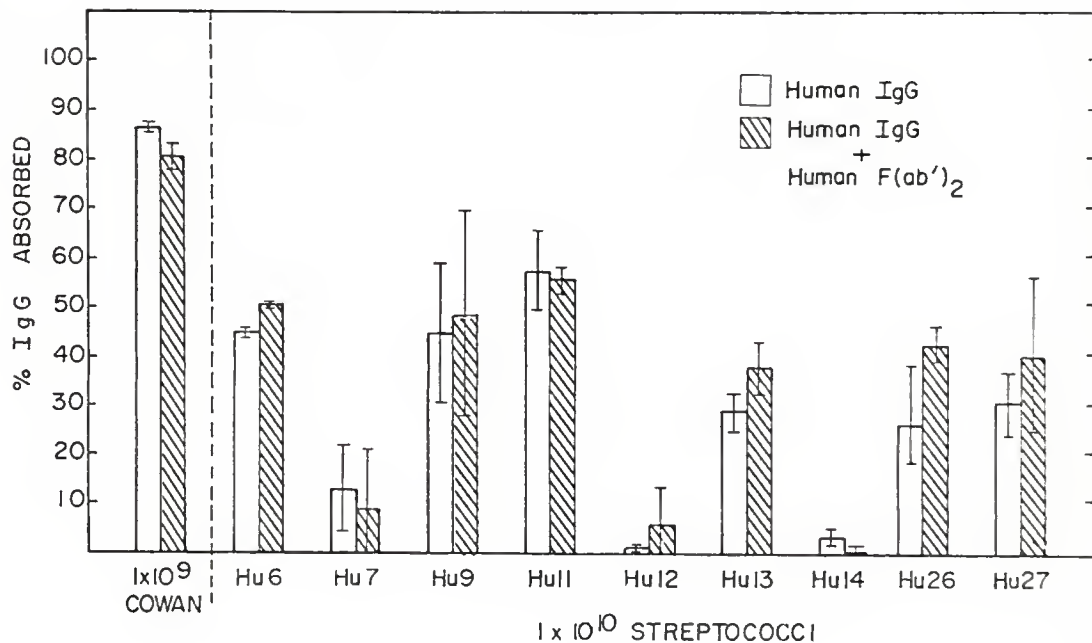
Figure 2-2. Adsorption of human IgG in the presence and absence of the
corresponding human IgG F(ab')$_2$ fragments.
Streptococci, 1 x 10^{10}, were mixed with 1 μg of human
IgG and 1 μg of human IgG plus an equimolar concentration
of the corresponding F(ab')$_2$ fragment. The amount of
whole IgG remaining after adsorption by the bacteria was
determined. Results are expressed as the percent of IgG
adsorbed, + the standard deviation. Hu6 is a group C
 ..streptococcus, the remainder belong to group A.
Adsorption by 1 x 10^9 <u>Staph</u>. <u>aureus</u> Cowan I is shown for
comparison.
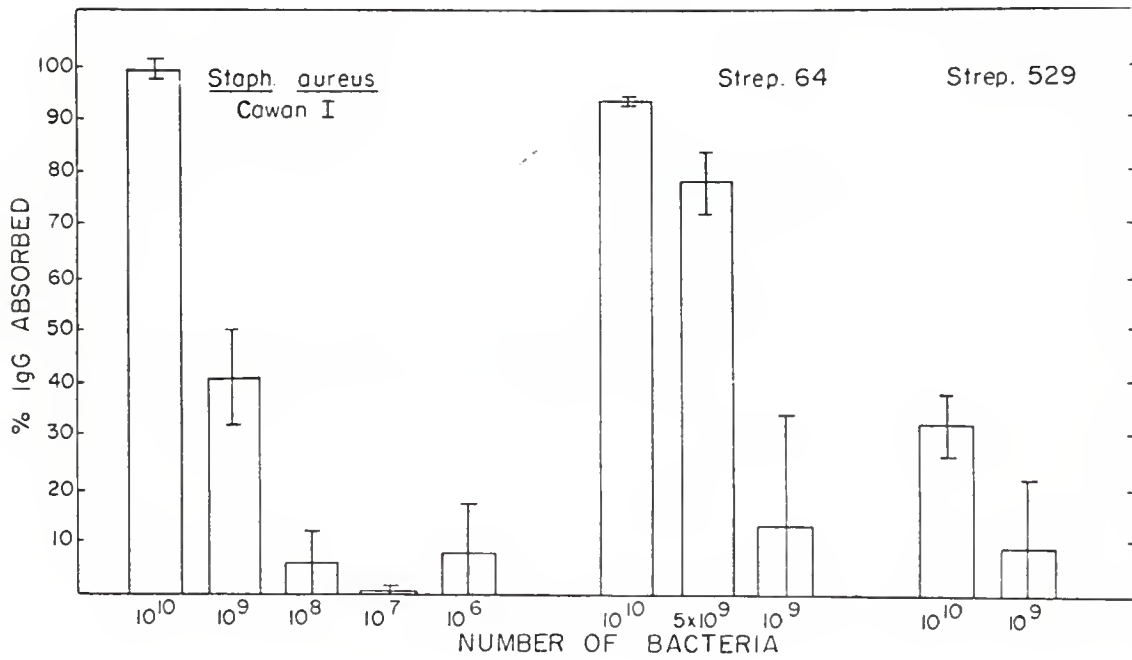
Figure 2-3. Adsorption of human IgG by varying numbers of bacteria.
Results are expressed as the percent of IgG adsorbed, +
the standard deviation.
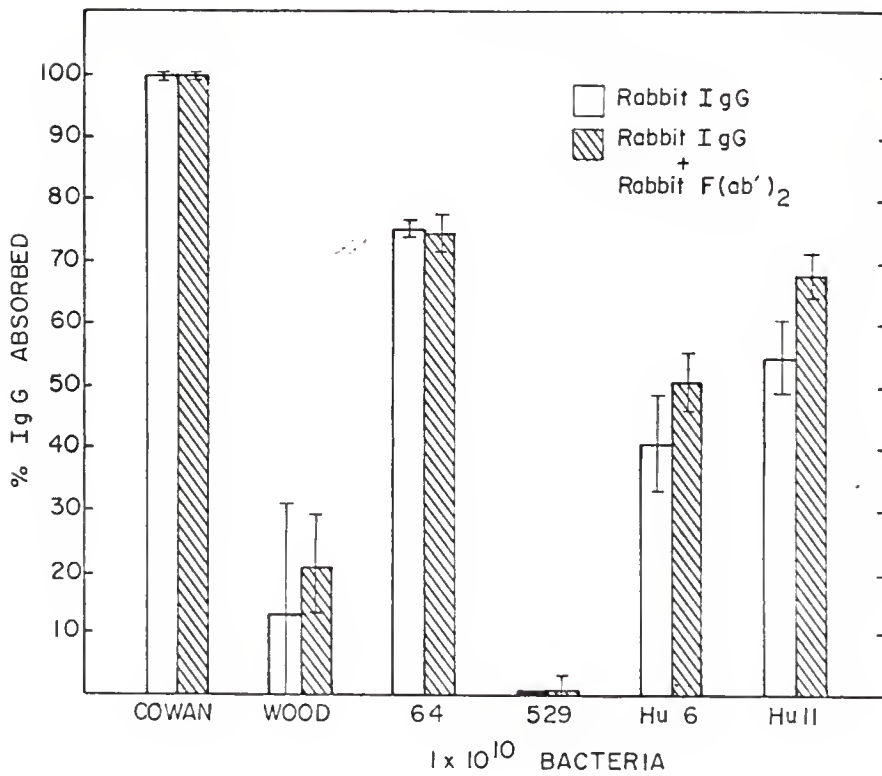
Figure 2-4. Adsorption of rabbit IgG in the presence and absence of the corresponding rabbit IgG F(ab')$_2$ fragments. <u>Staph. aureus</u> strains, Cowan I and Wood 46; group A streptococci strains 64, 529 and Hull and Group C streptococcus strain Hu6 were adsorbed with 1 μg rabbit IgG as described in Figure 2-2 and text.

Measurement of Fc-Receptors Released by Streptococci

To assess the role of Fc receptors in the pathogenesis of
bacterial infections it may be valuable to distinguish between cell
bound Fc-reactive material and that secreted by the bacteria during
growth. For this reason a rapid, reproducible method for detecting
Fc receptors in bacterial culture supernatants was sought. Langone et
al. (1977), have previously described a competitive binding assay that
could be used to measure nanogram quantities of IgG or protein A. This
assay was adapted to measure selectively protein A and related Fc
receptors. This has been achieved by carrying out the assay procedure
in two stages as outlined in Figure 2-5.

In the first stage 1 ml of test sample or buffer was mixed with
0.1 ml of a standard sample of agarose beads with covalently bound
rabbit IgG (Immunobead R-1 Bio-Rad Laboratories). The mixture is
incubated for 60 mins at 37°C, and then 2 mls of 0.01 M EDTA buffer
containing 0.1% gelatin, pH 7.4 is added and the Immunobeads pelleted
by centrifugation at 1,000 g for 5 mins. The supernatant is discarded
and the IgG beads, with any complexed Fc-reactive material, are washed
with 2 mls of the 0.01 M EDTA-gel buffer. One tenth of a milliliter of
$125I$ labeled protein A (approximately 20,000 cpm) and 1.0 ml of
VBS-gel are added to the washed beads and incubated an additional
60 mins at 37°C. At this time the beads are washed twice with 0.01 M
EDTA-gel buffer as described above and the quantity of $125I$ protein
A associated with the beads measured.

These assay conditions were found to be optimal from preliminary
kinetic studies. Under these assay conditions, maximal binding of
$125I$ protein A to the immunobeads was approximately 7,000 cpm. The

number of counts recovered when the assay was performed without

Immunobeads present (i.e., the background) was approximately 200 cpm.

By comparing the number of counts bound in the absence of cold protein

A to the number bound to the beads in the presence of known amounts of

unlabeled protein A (Pharmacia), the degree of inhibition can be

calculated and a standard curve obtained, Figure 2-6. The quantity of

Fc-reactive material in unknown samples can be measured by comparing

the observed percentage inhibition with the standard inhibition curve

and the results expressed in protein A equivalent units. For purified

protein A, 50% inhibition of binding was consistently observed with

solutions containing 10-20 ng/ml (see Figure 2-6). Similar results

were obtained when the assay was run in Todd Hewitt broth or in

Trypticase Soy Broth (data not shown). No inhibition was observed in

this two-stage assay with human IgG samples containing 100 μg/ml,

Figure 2-6. Non-specific interference from constituents of bacterial

culture media was observed when a one-stage assay for protein A or

protein A-like Fc receptors was used. This method was applied to

measuring secreted protein A-like material in the overnight culture

supernatant fluids of various streptococcal cultures. Of 15

β-hemolytic streptococci tested only two strains secreted detectable

levels of Fc-reactive material by the two-stage PA assay. The superna-

tant from an overnight culture of one group C strain (Hu6) contained

the equivalent of 10.2 $\pm$ 1.7 ng of protein A/ml. The supernatant from

an overnight culture of a group A strain (#64) contained the equivalent

of 5 $\pm$ 0.1 ng of protein A/ml. For comparison, overnight cultures of

Staphylococcus aureus Cowan strain and Wood 46 strain were cultured

under identical conditions as representative of high and low protein A

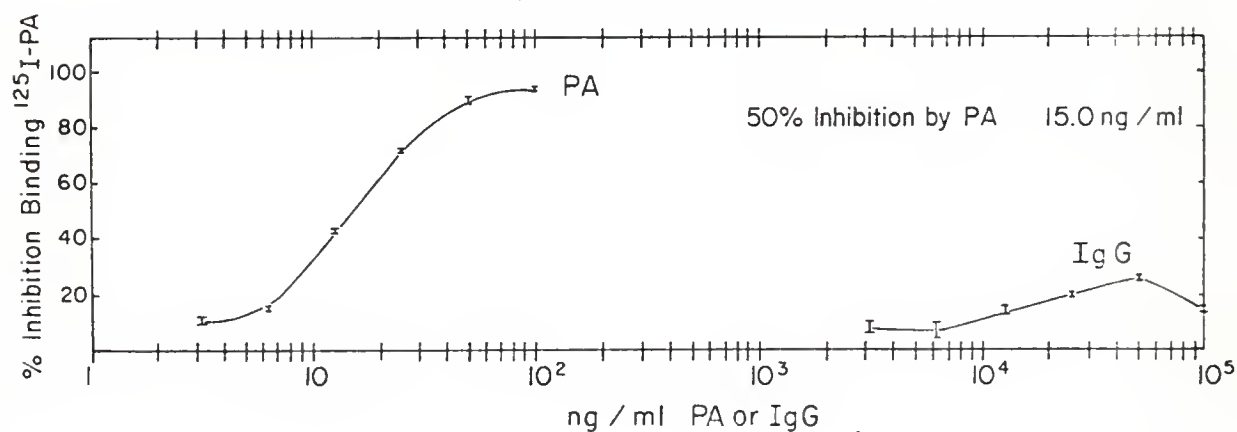Figure 2-5.   Two-stage assay selective for PA.

Figure 2-6. Standard Curve generated using purified PA in the
two-stage PA assay. Known quantities of cold purified PA
were tested for their ability to inhibit the binding of
^{125}I PA to IgG immunobeads as described in the text.
100 µg of human IgG did not significantly interfere in
this assay.

producers. The overnight culture supernatant from the Cowan strain contained 1850 ± 200 ng protein A/ml and the Wood strain supernatant contained 162 ± 7 ng PA/ml.

Any Fc receptors detected by this assay must bind to the IgG Fc region at or near the PA-binding site. To ensure that my results using streptococcal culture supernatants reflected absence of Fc receptors, rather than failure to detect the material due to binding at a site remote from the PA binding site, the ability of streptococcal culture supernatants to mediate agglutination of red cells with subagglutinating concentrations of antibody on their surface was tested as described in Materials and Methods. The culture supernatants were heated to 80°C prior to testing to destroy hemolysins that made this assay unworkable. [This method has previously been used to demonstrate soluble Fc-reactive material in streptococcal culture supernatants and extracts (Kronvall, 1973 and Havlíček, 1978).] I found that only the two streptococcal strains that contained detectable Fc receptors by the two-stage PA assay were capable of mediating agglutination of the sensitized red cells. The culture supernatants of the streptococcal strains Hu6 and 64 gave hemagglutination titers of 243 and 27 respectively and the Staphylococcus aureus strains Cowan I and Wood 46 had hemagglutination titers of 4,500 and 2,187 respectively.

Detection of Fc Receptors in Lancefield and Lysostaphin Extracts of Streptococci.

The results in the previous section suggest that despite the presence of relatively high levels of Fc receptors on the surface of certain streptococci these receptors were secreted only at low levels during culture. In order to determine if this assay could be used for streptococcal Fc receptors once solubilized or extracted from the

TABLE 2-1

PA-like Activity in Bacterial Extracts

Streptococcal Strain	Group	PA-like Material Extracted[a] ng PA Equivalent		
		Buffer Control	Lancefield Extract	Lysostaphin Extract
529	A	0	40.3	0.0
64	A	0	70.3	50.2
3706-T	A	0	0.0	0.0
H-1-JP	NG[b]	0	10.3	0.0
C 691	C	0	29.6	25.3

a = For details see text.
b = Not Grouped.

bacteria I tested for the presence of Fc receptors in Lancefield and lysostaphin extracts of streptococci which had surface Fc receptors as demonstrated by the IgG adsorption assay. The results presented in Table 2-1 demonstrate that Fc-reactivity could be detected in these extracts. This would suggest that the competitive binding assay would detect certain Fc receptors from streptococci if they had been secreted.

Discussion

In this chapter, methods for screening streptococci for the presence of surface Fc receptors and for measuring Fc-reactivities in bacterial culture fluids and extracts are described. The assay for measuring surface Fc receptors is based on the ability of differing numbers of streptococci to adsorb IgG in the presence or absence of corresponding $F(ab')_2$ fragments. This assay did not have any of the problems associated with assays involving ^{125}I labeled IgG. There was no significant nonspecific binding of IgG to the reaction vessel or difficulty distinguishing IgG bound through $F(ab')_2$ sites from that bound via the Fc region. Although the presence or absence of Fc receptors on a particular strain of bacteria was a consistent finding, the amount of IgG that could be adsorbed by Fc receptor positive strains varied from experiment to experiment. Since the reproducibility of adsorption was high for replicates within a culture, it is likely that this variation is due to differences in surface properties of bacteria obtained from different cultures. The method described was superior to the hemagglutination assay in sensitivity, objectivity, and reproducibility and was applicable to a wider range of streptococcal

strains, e.g., autoagglutinating bacteria. These studies demonstrated that certain clinical isolates of group A streptococci had 5-35% the level of Fc receptors of the protein A rich, Cowan strain Staphylococcus aureus, measured under identical conditions. This finding suggests that these strains would be a reasonable starting source for the extraction and characterization of a streptococcal protein A-like molecule.

Additionally, this assay can be used to characterize the species of IgG with which streptococcal Fc-reactive material would react (see Figures 2-3 and 2-4). For example, streptococcus 529 effectively adsorbed human IgG but did not adsorb rabbit IgG (compare Figures 2-3 and 2-4). Langone (1978) has shown that the competitive binding assay for IgG can be used to quantify both protein A-reactive and nonreactive species of IgG, the methods described in this study could be expanded to examine the reactivity of bacteria with any species, class or subclass of immunoglobulin.

The assay for soluble Fc-reactive material described is an extension of the competitive binding assay for IgG and protein A previously described by Langone et al. (1977). By carrying out the assay in two stages, it was made selective for PA-like Fc-reactive material and could be carried out in culture media without loss of sensitivity. The sensitivity of this procedure was not affected by the presence of bacterial hemolysins. In agreement with previous reports, secreted protein A from staphylococci could be readily detected (Winblad and Ericson, 1973) but only a few streptococci secreted Fc receptors. In those strains the levels of Fc-reactive material secreted were close to the limit of detection of the assay system. Significant levels of

Fc-reactive material could be detected in Lancefield and lysostaphin extracts of certain group A streptococci using this assay. Although this method is based on direct competition of ^{125}I protein A for its receptor site on the Fc region of IgG, I was unable to find a streptococcal culture fluid that could mediate hemagglutination which was not positive in the PA binding assay.

The assays for cell-bound and soluble Fc receptors represent improved methods for screening bacteria for the presence or production of these biologically active molecules. These methods are used as the basis for the remainder of my work to detect and characterize both cell surface and soluble Fc receptors.

CHAPTER THREE
DETERMINATION OF THE STRUCTURAL RELATIONSHIP BETWEEN
STAPHYLOCOCCAL PROTEIN A AND STREPTOCOCCAL Fc RECEPTORS

Introduction

In Chapter 2 methods for identifying streptococci with surface Fc receptors were described. In this chapter I have used a monospecific polyclonal antibody to protein A prepared in chickens to determine whether the Fc receptor(s) on a number of strains of Staphylococcus aureus and group A and group C streptococci are related antigenically to protein A, or represent distinct surface moieties with a common functional activity, namely the ability to bind to the Fc region of IgG. This approach has proved valuable in establishing total or partial identity between bacterial surface antigens that share a common functional activity, e.g., the anti-phagocytic M protein of group A streptococci that is known to exist in a variety of antigenic forms (Ferrieri, 1975). The purpose of this study was to ensure that any streptococcal Fc receptor I attempted to isolate was distinct from protein A.

Materials and Methods

Bacteria Strains, Media and Growth Conditions

Staphylococcus aureus Cowan I strain and human isolates of Staphylococcus aureus and β-hemolytic streptococci of groups A and C

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were used. Media and growth conditions were as described in
Chapter 2.

Iodination of Protein A

Purified protein A (Pharmacia) was iodinated by the mild lactoper-
oxidase method using enzyme beads (Bio-Rad) (Morrison et al., 1971), as
described in Chapter 2.

Chicken Anti-Protein A

Monospecific antiserum to staphylococcal protein A was a gift from
Dr. John Langone, National Cancer Institute, Bethesda, Maryland. This
antiserum was prepared by immunizing roosters using the following
schedule. Preimmunization blood samples were obtained five days prior
to the initial injection. Roosters were injected with 50 μg of protein
A (Pharmacia) intramuscularly (I.M.) in complete Freund's adjuvant.
Blood samples were obtained one week later to test for antibody
production. Four weeks after the initial injection, the roosters were
boosted I.M. with 50 μg of protein A in incomplete Freund's adjuvant
and serum collected one week later. The resulting antiserum gave a
precipitin line against purified protein A in a double immunodiffusion
assay. No line was observed when preimmunization serum was used,
confirming that the reaction was not mediated via the Fc region of
chicken immunoglobulins. All of the anti-protein A activity could be
removed by passage over protein A immobilized on Sepharose. The
ability of the antiserum to precipitate ^{125}I protein A could be
completely inhibited by cold protein A, either in the purified form
(Pharmacia), or as extracts of Staphylococcus aureus Cowan I strain.

Human IgG and IgG Assay

Human IgG was prepared by chromatography of normal human serum on DEAE cellulose (Boyle and Langone, 1980) and quantified by the competitive binding assay of Langone et al. (1977) as described in Chapter 2.

Selection of Bacteria with Surface Fc receptors

Fc receptors on the surface of bacteria were detected by the ability of bacteria to adsorb human IgG from solution. The details of this assay have been described fully in Chapter 2.

Using this assay four clinical streptococcal isolates with surface Fc receptors were selected. Three of these strains are group A (9, 64/14, and 11) and one Group C (Hu6). The ability of these bacterial strains to adsorb IgG was described in Chapter 2.

Results

Competitive Binding Assay for the Quantitation of Antibodies Specifically Directed Against Staphylococcal Protein A

A competitive binding assay was developed to quantify antibody to protein A. In this assay the antigen combining sites on chicken anti-protein A antibody compete with the Fc region of IgG immobilized on agarose beads (Bio-Rad) for radio iodinated protein A. Two tenths of a milliliter of anti-protein A diluted in veronal buffered saline, pH 7.4, containing 0.1% gelatin (VBS-gel) is mixed with 0.1 ml of a standard suspension of agarose beads containing immobilized rabbit IgG (Bio-Rad) and 0.1 ml of iodinated PA containing approximately 20,000 cpm. Following a 1.5 hr incubation at 37°C, 2 mls of EDTA-gel are added to each tube. The tubes are centrifuged at 1,000 g for 5 mins and the supernatant fluid is decanted. Following a second wash with an

additional 2 mls of EDTA-gel as described, the amount of ^{125}I

protein A adhering to the beads is determined in an LKB Gamma Counter.

Maximal binding of ^{125}I protein A to the Immunobeads was

approximately 6,000 cpm. The number of counts recovered when the assay

was performed without Immunobeads present (i.e., the background) was

approximately 200 cpm. By comparing the number of counts bound in the

absence of chicken anti-protein A to the number of counts bound to the

beads in the presence of various dilutions of chicken anti-protein A,

the degree of inhibition of protein A binding can be calculated and a

standard curve relating antibody concentration to inhibition can be

obtained. The assay is summarized in Figure 3-1A and a typical

inhibition curve is shown in Figure 3-1B.

Under the conditions of the assay a 1:10,000 dilution of the anti

protein A antiserum routinely inhibited the binding of ^{125}I protein

A to Immunobeads by 50%. No inhibition of ^{125}I protein A binding

to Immunobeads was observed in the presence of a 1:10 dilution of

preimmunization chicken serum, confirming that protein A is not

reactive with the Fc region of chicken immunoglobulins (Langone,

1978).

Detection of Protein A on the Surface of Bacteria

In these studies the protein A positive Staphylococcus aureus

Cowan I strain and four Fc receptor positive streptococcal strains were

tested for their ability to react with anti-protein A antibody via the

(Fab')$_2$ region, by adsorbing a standard dilution of the anti-protein

A antibody with various numbers of bacteria (Figure 3-2). In this

adsorption assay, differing numbers of bacteria were mixed with 1 ml of

a 1:2,000 dilution of chicken anti-protein A for 90 mins at 37°C and

COMPETITIVE BINDING ASSAY
FOR THE DETECTION OF ANTI − PROTEIN A
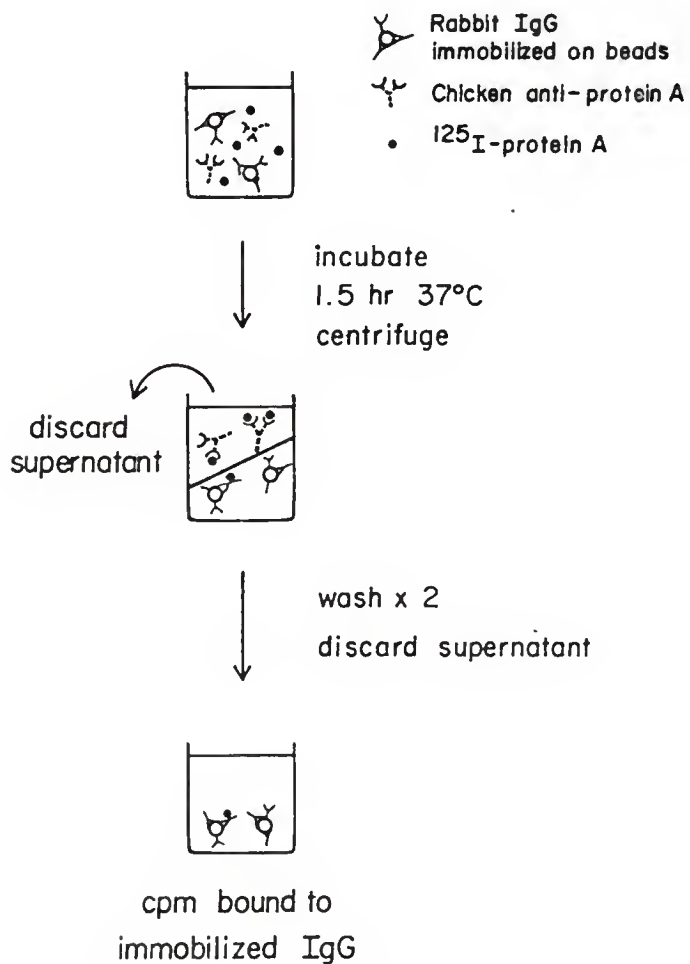
Figure 3-1.   Competitive binding assay for quantitation of chicken
anti-protein A antibody.
A.   Flow chart of assay.
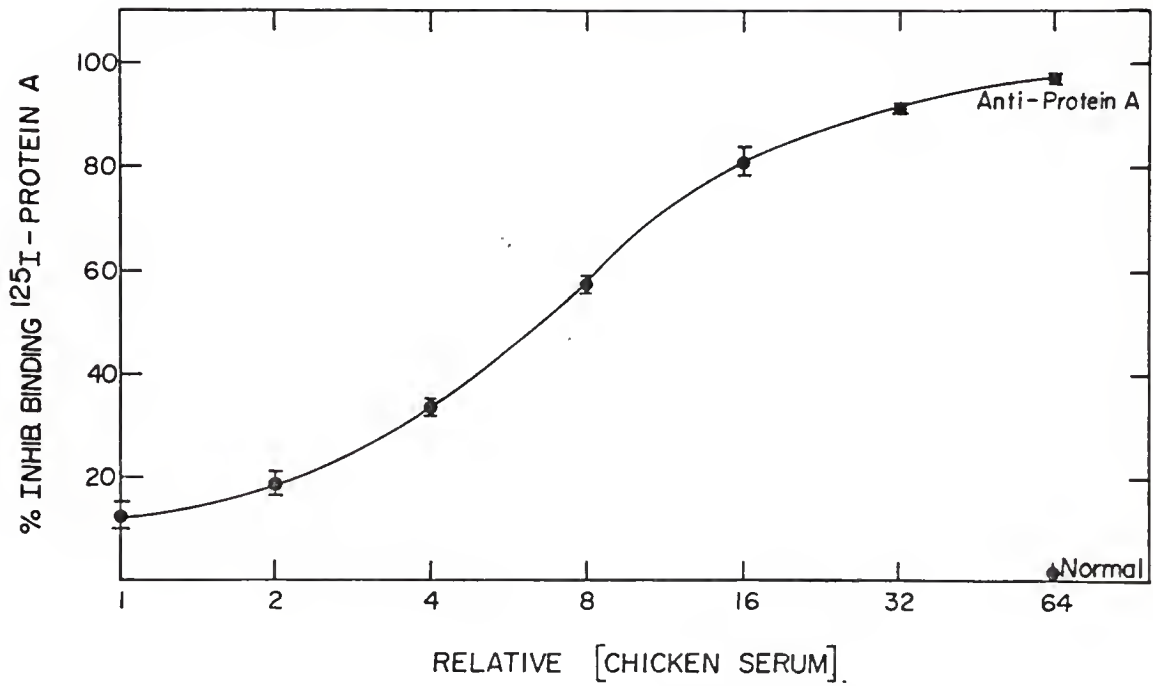
Figure 3-1. (continued)
B. Typical standard curve obtained using the chicken
anti-protein A antiserum. A relative concentration of 1
represents a 1 to 64,000 dilution of the chicken
anti-protein A antiserum. The results are presented as
percent inhibition of ^{125}I protein A binding ± the
standard deviation. For precise experimental details see
text.

centrifuged. Duplicate samples of an aliquot of the supernatant fluids were tested for residual anti-protein A using the assay described above. The percent anti-protein A adsorbed was determined by comparing the amount of anti-protein A in the adsorbed supernatant fluids to control tubes containing anti-protein A and no bacteria. Results are expressed as the percent of anti-protein A adsorbed by various numbers of bacteria (Figure 3-2). Adsorption of anti-protein A by Staphylococcus aureus Cowan I strain was not affected by the presence of a 1:10 dilution of preimmunization chicken serum (data not shown), confirming that normal chicken immunoglobulin is nonreactive with protein A.

These results demonstrate that chicken anti-protein A is able to bind antigenically with a protein A-bearing-bacteria strain and the amount adsorbed is dependent on the number of bacteria used for adsorption (Figure 3-2A). None of the IgG Fc-reactive streptococcal strains were capable of adsorbing anti-protein A, even though these streptococci were able to adsorb equivalent amounts of human IgG at the concentrations of bacteria tested. These findings indicate that the Fc receptors on streptococci are not antigenically related to protein A. By contrast the results with Staphylococcus aureus Cowan strain would be consistent with the Fc receptor on these bacteria being protein A or being co-expressed along with protein A.

The following experiment was designed to examine whether pretreatment of the Cowan strain bacteria with anti-protein A antibody would block its ability to adsorb human IgG via the Fc region.

STAPH. AUREUS COWAN   STREPTOCOCCI
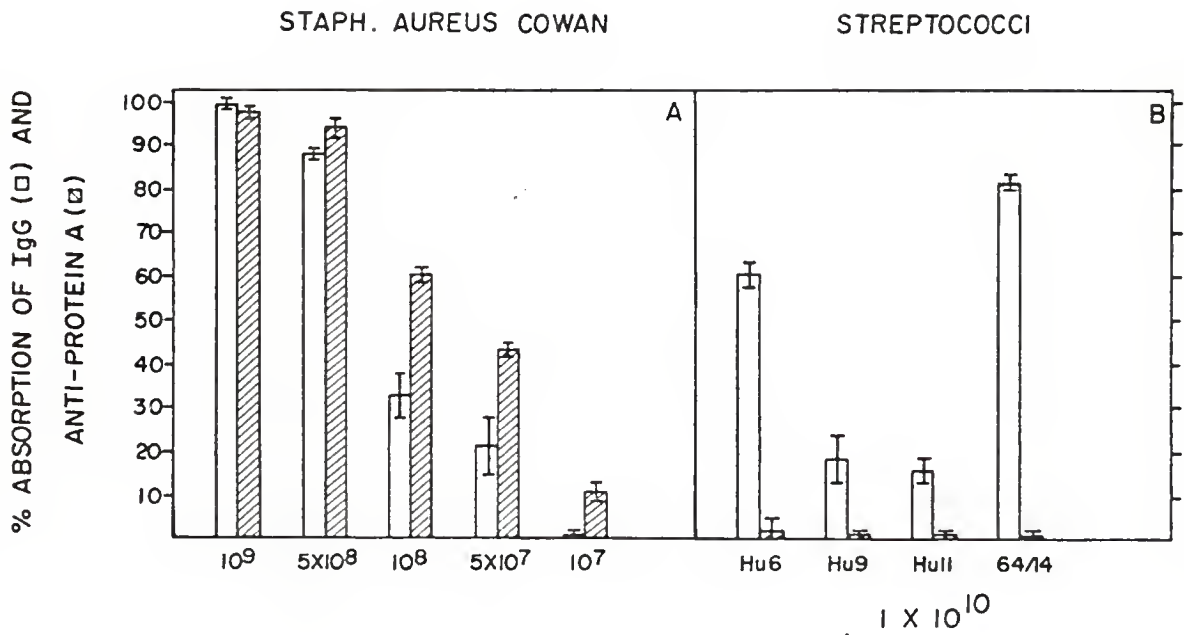
Figure 3-2.   Ability of Staphylococcus aureus Cowan I (A) and various
               streptococci (B) to selectively interact with the
               Fc-region of human IgG (☐) or with the Fab₂ region of
               chicken anti-protein A antibody (▨). Results are
               presented as percent IgG or of antibody to protein A
               absorbed ± standard deviation. For precise experimental
               details see text.

Blocking of Fc receptor Activity on Staphylococcus aureus Cowan Strain by Pretreatment with anti protein A

Aliquots of a standard suspension of Staphylococcus aureus Cowan strain that would adsorb approximately 350-400 ng of human IgG from solution were tested for their ability to remove IgG from solution following treatment of the bacteria with anti protein A antibody. A standard number of bacteria was incubated with 1 ml of various dilutions (1:125 to 1:4,000) of chicken anti-protein A or normal chicken serum as described above for the anti-protein A adsorption assay. Following an incubation of 1 hr at 37°C, the bacteria were washed twice with 2 mls of 0.01 M EDTA-gel to remove any unbound anti-protein A. One milliliter of VBS-gel containing 500 ng of human IgG was added to each bacteria pellet and to control tubes containing no bacteria. Following a 1 hr incubation at 37°C, all tubes were centrifuged and aliquots of the supernatants were tested for the amount of IgG adsorbed as described earlier. The Fc receptor positive group C streptococcus (Hu6) which failed to react with the anti-protein A antibody was included as a negative control, (see Figure 3-2). The results in Figure 3-3A show that the adsorption of IgG was completely inhibited when the Cowan I strain was preincubated with high concentration of anti-protein A antiserum, but not by equivalent concentrations of normal chicken serum. Pretreatment of the group C streptococcus with anti-protein A did not affect its ability to subsequently adsorb human IgG (see Figure 3-3B).

Comparison of Fc-receptors on the Clinical Staphylococcus aureus Isolates to those of the Laboratory Cowan Strain

The presence of protein A on other strains of staphylococci has been suggested by their ability to react with the Fc region of IgG. Since certain streptococci share this property using a receptor
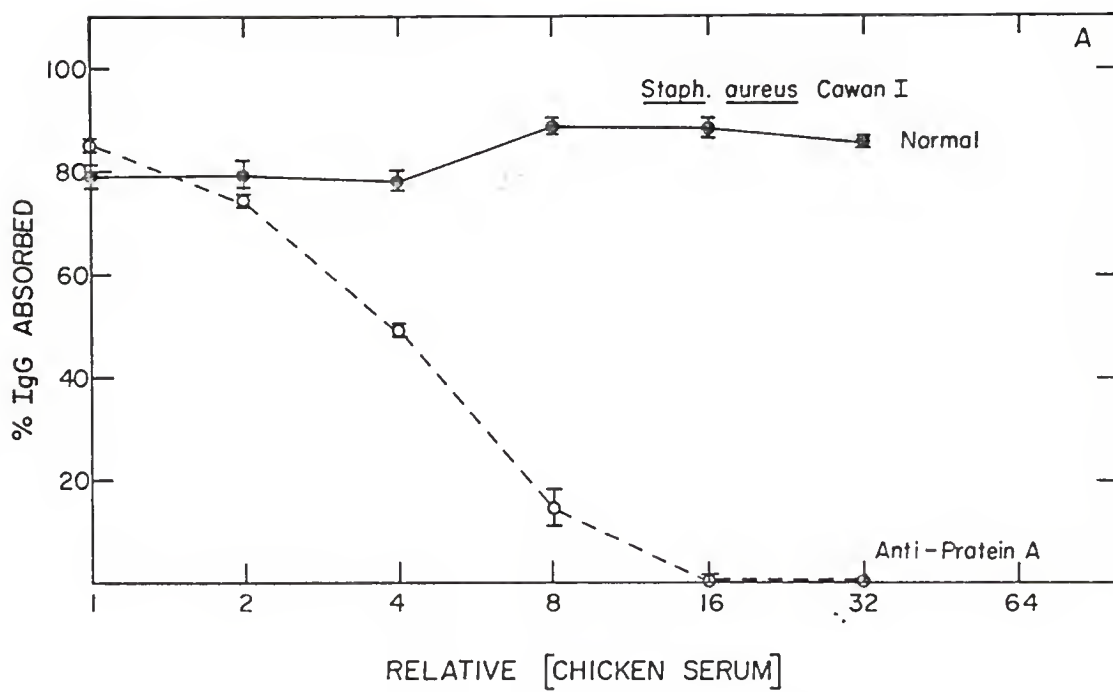
Figure 3-3. Binding of human IgG to bacteria pretreated with chicken
anti-protein A anti-serum or normal chicken serum.
A. Results using Staphylococcus aureus Cowan I.
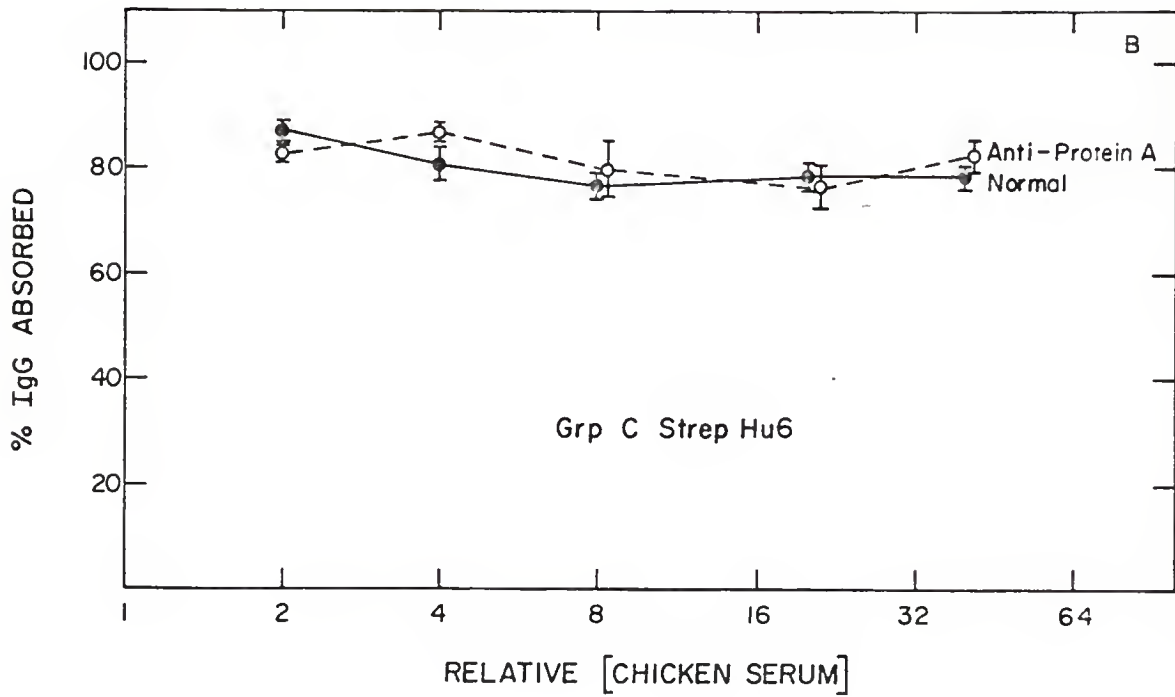
Figure 3-3. (continued)
B. Results using a representative streptococcal strain.
In this case a human clinical group C isolate designated
Hu6.
    For both A and B a relative concentration of 1 for
chicken anti-protein A anti-serum or the preimmunization
chicken serum (normal) represents a 1 to 4,000 dilution.
Results are expressed as the percent IgG absorbed +
standard deviation. For precise experimental details see
text.

antigenically distinct from protein A, in the next series of experiments I determined whether all staphylococcal Fc reactivity was mediated by protein A-like molecules or if differing Fc receptors could be defined on the surface of various staphylococcal strains other than the Cowan strain.

Clinical isolates of Staphylococcus aureus were screened for their ability to adsorb human IgG from solution. As expected, a range of Fc-reactivities was observed with certain of the strains being capable of adsorbing equivalent quantities of IgG to the Cowan strain on a per bacterium basis, while other strains were less effective. Of the nine isolates screened, all were capable of adsorbing significant quantities of IgG under the standard assay conditions (data not shown). From these preliminary screening experiments, the staphylococcal isolates were adjusted to yield approxiamtely equivalent levels of IgG adsorbing capacity by varying the number of bacteria, Table 3-1. The ability of these bacteria to remove IgG when preincubated with buffer, normal chicken serum or chicken anti-protein A was tested. For these studies a single concentration of antibody (1:250) was selected, based on the results obtained in Figure 3-3. In each case the ability of clinical isolates of Staphylococcus aureus to adsorb human IgG could be entirely blocked by preincubation in chicken anti protein A, Table 3-1. No inhibition was observed when the bacteria were pretreated with normal chicken serum, Table 3-1. These observations indicate that despite the obvious quantitative differences in IgG binding capacity of the staphylococcal isolates tested, the Fc receptor was a protein A-like molecule in all cases. Four streptococcal strains, two group A and two group C, which demonstrated the ability to adsorb IgG were included.

TABLE 3-1

Effects of Specific Chicken Anti-Protein A Antibody
on the Ability of Bacteria to Adsorb IgG[a]

Genera	Strain	Number Bacteria	% IgG adsorbed		
			Buffer	Normal Chicken Serum 1:250	Chicken Anti-Protein A 1:250
Staph. aureus	Cowan	5×10^8	$81 + 8$	$88 + 2$	<5
	Hu 16	10^9	$67 + 5$	$83 + 1$	<5
	Hu 17	2.5×10^8	$48 + 7$	$46 + 4$	<5
	Hu 18	10^9	$69 + 4$	$55 + 4$	<5
	Hu 19	5×10^8	$79 + 1$	$71 + 1$	<5
	Hu 20	10^{10}	$62 + 6$	$69 + 4$	<5
	Hu 21	10^9	$74 + 3$	$79 + 1$	<5
	Hu 22	2×10^8	$66 + 1$	$50 + 3$	<5
	Hu 23	2.5×10^8	$48 + 1$	$48 + 7$	<5
	Hu 25	10^{10}	$88 + 1$	$89 + 1$	<5
Strep. Grp. C	26RP66	10^9	$88 + 2$	$89 + 1$	$89 + 1$
	Hu 6	10^{10}	$92 + 1$	$94 + 1$	$88 + 1$
Strep. Grp. A	Hu 9	10^{10}	$39 + 8$	$39 + 8$	$33 + 1$
	64/14	10^{10}	$90 + 2$	$96 + 4$	$83 + 4$

a = Bacteria were preincubated in buffer, in normal chicken serum, or in chicken anti-protein A antiserum, for 1 hr at ambient temperature. The bacteria were washed twice and their ability to remove human IgG from solution was tested.

Preincubation of these streptococcal strains with either anti-protein A

or normal chicken serum did not inhibit the adsorption of IgG

(Table 3-1).

Discussion

The purpose of this part of the study was to determine whether all

bacterial Fc receptors were structurally related to protein A, or if

distinct Fc receptors were present alone or together with protein A on

the surface of other bacteria. The results presented in this chapter

clearly demonstrate that the Fc receptor(s) on four different

streptococcal strains were not antigenically related to staphylococcal

protein A. By contrast, the reactivity of all Fc receptors on

Staphylococcus aureus Cowan strain could be totally inhibited by

pretreating the bacteria with anti-protein A antibody. Studies with

nine fresh clinical isolates of Staphylococcus aureus demonstrated

differences in the quantity of Fc receptors expressed on their surface

(see Table 3-1). In all cases, a close correlation was observed

between their ability to bind anti-protein A antibody and to adsorb IgG

via the Fc region. Pretreatment of any of these bacterial strains

with anti-protein A antibody totally inhibited their ability to react

with the Fc region of human IgG. These results would suggest that all

staphylococcal Fc receptors were protein A-like and that a subgroup of

non-protein A Fc receptors could not be detected.

The results presented in this chapter support the idea that there

are different classes or types of Fc receptors on staphylococci and

streptococci (Myhre and Kronvall, 1981b). They do not exclude the

possibility that the region of the receptor molecule that directly

interacts with the Fc region of IgG may not be immunogenic or is weakly immunogenic. Such a region would be equivalent to the antigenic combining region of an immunoglobulin and would only be detected by the equivalent of an anti-idiotypic antibody. Although the exact nature of the combining site in the Fc receptor molecule that binds to the Fc region of IgG has not been elucidated, I have shown that the Fc reactivity on streptococci is not mediated by protein A or a closely related molecule. By contrast, all Fc binding activity associated with staphylococci was shown to be protein A-like in nature.

Using the methods described in Chapters 2 and 3, the next part of this study was to attempt to solubilize and isolate a non-protein A-like streptococcal Fc receptor.

CHAPTER FOUR
ISOLATION AND PARTIAL CHARACTERIZATION
OF THE Fc RECEPTOR FROM A GROUP C STREPTOCOCCUS

Introduction

Attempts to extract and purify streptococcal Fc receptors have met
with limited success. Unlike protein A none of the streptococcal Fc
receptors are secreted in significant quantities during culture
(Kronvall, 1973; Schalén, 1982). A variety of extraction procedures
have been tested including phage lysis (Christensen and Holm, 1976),
heat (Christensen and Holm, 1976; Christensen and Kronvall, 1974) or
treatment with hot acid or hot alkali (Havlíček, 1978; Schalén et al.,
1978; Christensen et al., 1979b). In most reports the yield of soluble
Fc receptor was low. The most highly characterized streptococcal Fc
receptor was isolated by Grubb et al. (1982) from a group A strepto-
coccus following alkaline extraction. This receptor was heterogeneous
in size with the predominant activity having a molecular weight of
29,500 daltons and was only obtained when protease inhibitors were
included during purification.

Using the techniques outlined in Chapter 2, I was able to identify
a group C streptococcus with Fc binding capacity approximately equiva-
lent to the adsorbing capacity of the protein A-rich Staphylococcus
aureus Cowan I strain. The receptor on this group C streptococcus was
shown to be antigenically distinct from protein A (Chapter 3). This
chapter describes the isolation in high yield of a functionally

homogenous Fc receptor from this group C streptococcus, which has been designated FcRc.

Materials and Methods

Bacteria and Bacteriophage

The β-hemolytic group C streptococcal strain designated 26RP66 and the Cl bacteriophage were a gift from Dr. Vincent Fischetti of the Rockefeller University, New York, New York. This strain was selected based on its high surface Fc receptor activity as determined by immunoassay as described in (Chapter 2). For all of the studies bacteria were grown in Todd Hewitt broth and phage lysis was carried out using a modification of the procedure of Fischetti et al. (1971).

Phage-associated Lysin Activity

Phage-associated lysin activity was detected by the lysis of a group A streptococcal strain as described by Fischetti et al. (1971).

Extraction of Fc receptors

The streptococcal strain 26RP66 was grown overnight in 3 liters of Todd Hewitt broth. A bacterial pellet was recovered by centrifugation and washed once in phosphate buffer saline (PBS) pH 7.4. Aliquots containing approximately 0.25 g of bacteria (wet weight) were enzyme extracted into 6 ml of appropriate buffer containing 100 μg DNAse under the following conditions:

1) Mutanolysin (Miles) extraction was carried out using 2,000 units of enzyme in 0.05 M KH_2PO_4, pH 6.5 (Siegel et al., 1981).

2) Pepsin (Sigma) extraction was carried out using 1,750 units in 0.05 M KH_2PO_4, pH 5.8 (Manjula and Fischetti, 1980).

3) Lysozyme (Sigma) extraction was carried out using 24,000 units in 0.05 M KH_2PO_4, pH 6.3 (Forsgren, 1969).

4) Lysostaphin (Sigma) extraction was carried out using 175 units in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 (Sjöquist et al., 1972).

These conditions were previously used to isolate either streptococcal cell wall constituents or staphylococcal protein A (Forsgren, 1969; Sjöquist et al., 1972; Manjula and Fischetti, 1980; Siegel et al., 1981). All extractions were carried out for 4 hrs at 37°C. The extracts were then centrifuged at 10,000 g for 15 mins and the supernatants recovered, dialyzed against PBS, and stored at 4°C until tested for functional Fc receptor activity by the method described below. Detergent extraction was carried out in a similar way using 1% Tween-20 in 0.15 M PBS, pH 7.4.

Hot Acid/Hot Alkaline Extracts

Hot acid/hot alkaline extracts were carried out according to the method of Lancefield (1928). Bacteria, 0.25 g, were suspended in 3 mls of 0.15 M PBS and the pH was adjusted to 2.0 (or 10) with 0.5 M HCl (or 0.5 M NaOH). The bacterial suspension was boiled for 10 mins and the pH was neutralized. The final volume was adjusted to 6.0 mls and the supernatants recovered as described above.

IgG, IgG Fragments and Immobilized IgG Preparations

Stock human IgG was prepared by chromatography of normal human or rabbit serum on DEAE cellulose (Boyle and Langone, 1980). Aliquots were stored at -70°C until use. The $F(ab)_2$ fragments of human IgG were prepared by pepsin digestion as described in (Chapter 2). Immunoglobulin G was immobilized to Immunobeads (Bio Rad, Richmond,

California) for use in the competitive binding assay as described by Langone et al., 1979a.

Immobilized IgG for affinity purification of the streptococcal Fc receptor was prepared by covalently coupling human IgG to the high capacity Affi-gel 15 activated beads (Bio-Rad, Richmond, California). Ten milligrams of gel washed with 3 volumes of isopropanol and 3 volumes of deionized water was mixed with 10 ml of human IgG containing 7.3 mgs IgG/ml. The coupling reaction was carried out in 0.1 M HEPES, pH 7.5 at 4°C overnight with gentle rocking. Unreacted sites were blocked by the addition of 0.1 ml of 1 M ethanolamine HCl, pH 8.0 for each ml of gel. One hour was allowed for complete blocking and the IgG coupled gel was extensively washed in VBS gel and stored at 4°C in VBS gel containing 0.02% sodium azide. Prior to use the immobilized IgG was washed with 10 volumes of glycine-HCl, pH 2.0 and reequilibrated in phosphate buffered saline, pH 7.4.

Iodination of PA and the Streptococcal Fc-receptor

Purified PA (Pharmacia) and the streptococcal Fc receptor (FcRc) were iodinated by the mild lactoperoxidase method using enzyme beads (Bio-Rad) (Morrison et al., 1971), as described in Chapter 2.

Detection of Soluble Fc-receptors

The method developed for the detection of soluble Fc receptors in extracts is described in (Chapter 2). Essentially, this competitive binding assay measures the ability of Fc receptors to inhibit binding of either ^{125}I labeled protein A or ^{125}I labeled Fc receptor to immobilized human or rabbit IgG. In the initial part of this study, ^{125}I protein A was used and one unit of Fc receptor activity was defined as the concentration of material that would inhibit its binding

by 30% under standard assay conditions. Once streptococcal Fc

receptors had been isolated and labeled, they were used for the assay

and an absolute value in ng/ml was assigned based on the inhibition of

affinity purified standards included in each assay. The absolute

protein concentration of the standard was determined using the Bio-Rad

protein assay (Richmond, California), which is a modification of

Bradford's method (Bradford, 1976).

Polyacrylamide Gel Electrophoresis

FcRc preparations containing 15-60 μg of unlabeled material or

1.7×10^5 cpm of iodinated material were applied to 7% polyacrylamide

disc gels. Samples were electrophoresed at 1.5 mamp per gel in 0.025 M

Tris, 0.2 M glycine, pH 8.3 until the tracking dye was 1 cm from the

bottom of the gel. Gels were either fixed and stained with Coomassie

Brilliant Blue or frozen and sliced into 1 mm sections. Gel slices

were either counted for radioactivity on an LKB gamma counter or eluted

for 72 hrs into VBS-gel and tested for functional activity. Samples

were also run on 0.1% SDS, 7% polyacrylamide gels as described above

with two exceptions. First, all samples were boiled for 1 min in 2%

SDS prior to electrophoresis. Second, the electrophoresis buffer was

0.1% SDS, 0.025 M Tris, 0.2 M glycine pH 8.3. Molecular weight

standards (Sigma, St. Louis) were included in each SDS polyacrylamide

gel assay. Myosin (200,000 daltons), β-galactosidase (116,000

daltons), phosphorylase b (94,000 daltons), bovine serum albumin

(68,000 daltons), egg albumin (43,000 daltons), carbonic anyhydrase

(30,000 daltons), β-lactoglobin (18,400 daltons).

Preparation and Measurement of Chicken Anti-FcRc

Antibody to the streptococcal FcRc was prepared by immunizing white leghorn hens using the following schedule. Preimmunization samples were obtained two days prior to the initial injection. Chickens were injected with 25 μg of FcRc intramuscularly (I.M.) in complete Freund's adjuvant. Blood samples were obtained two weeks later to test for antibody production. Two weeks and five weeks after the initial injection, the chickens were boosted I.M. with 25 μg of FcRc in incomplete Freund's adjuvant. Serum was collected one week after the final injection.

Competitive Binding Assay for the Quantitation of Antibodies

Antibodies to streptococcal FcRc were measured by a modification of the competitive binding assay described in (Chapter 3). In this assay the antigen combining sites on chicken anti-FcRc antibody compete with the Fc region of human IgG immobilized on agarose beads (Bio-Rad) for radio iodinated FcRc. Antiserum to FcRc was diluted in veronal buffered saline, pH 7.4, containing 0.1% gelatin (VBS-gel) and 0.2 ml of the dilution was mixed with 0.1 ml of a standard suspension of immobilized human IgG beads and 0.1 ml of iodinated FcRc containing approximately 30,000 cpm. Following a 1.5 hr incubation at 37°C, 2 ml of EDTA-gel are added to each tube. The tubes were centrifuged at 1,000 g for 5 mins and the supernatant fluid decanted. Following a second wash with an additional 2 ml of EDTA-gel, the amount of ^{125}I FcRc adhering to the beads was determined in an LKB Gamma Counter. Maximal binding of ^{125}I FcRc to the Immunobeads was approximately 6,000 cpm. The number of counts recovered when the assay was performed without Immunobeads present (i.e., the background) was approximately

200 cpm. By comparing number of counts bound in the absence of chicken anti-FcRc to the number of counts bound to the beads in the presence of various dilutions of chicken anti-FcRc, the degree of inhibition of FcRc binding can be calculated and a standard curve relating antibody concentration to inhibition can be obtained. No inhibition of binding of ^{125}I FcRc was observed in the presence of preimmunization chicken serum.

Results

Solubilization of Fc Receptor

The group C streptococcal strain designated 26RP66 was selected because of its high level of surface Fc receptors. A variety of extraction procedures were tested including phage lysis, alkaline extraction, acid extraction, detergent extraction, enzyme treatment with pepsin, lysostaphin, lysozyme or mutanolysin. The resulting cell free lysates were tested for soluble Fc receptors using the competitive binding assay described in the Methods. The only treatments that resulted in significant quantities of soluble Fc receptor activity were phage lysis (approximately 5×10^4 units/g bacteria extracted), mutanolysin treatment (approximately 3×10^4 units/g bacteria extracted) and treatment with hot acid (approximately 2×10^3 units/g bacteria extracted). Extraction of the bacteria with detergent, hot alkali, lysozyme or lysostaphin did not solubilize detectable quantities of a functionally active Fc receptor. The extracts were compared by three criteria: 1) the total yield of Fc receptor recovered/unit weight of bacteria, 2) the specific activity calculated as the soluble FcR activity divided by the OD_{280} of the extract

and 3) the charge heterogeneity of functional activity. This was measured following elution from non-denaturing polyacrylamide gels. The material obtained by phage lysis of bacteria demonstrated the highest yield, highest specific activity and was among the least heterogeneous of the extracts. Consequently I chose to pursue the solubilization of Fc receptors using phage lysis.

Isolation of Fc Receptors from the Supernatant of a Phage-Lysed Group C Streptococcal Culture

The group C streptococcus was grown to an OD_{650} of 0.3 in Todd Hewitt broth. To this culture was added approximately 3×10^{12} pfu of C1 bacteriophage/liter of culture and the bacteria allowed to lyse. After lysis was complete EDTA was added to a final concentration of 0.05 M and DNase to a final concentration of 0.5 μg/ml. The resulting supernatant which was filtered through a sinter glass filter was shown to contain two soluble activities: 1) an Fc receptor activity and 2) a bacteriolytic enzyme activity - the phage associated lysin originally described by Fischetti et al. (1971). The supernatant contained no detectable protease activity and the Fc receptor activity was found to be stable for over a month at 4°C or in excess of six months at -70°C. The crude supernatant was concentrated 30 fold using a Millipore Pellicon concentrator with a molecular weight cut off of 10,000 daltons. Residual cellular debris was removed by centrifugation at 27,000 g for 2 hrs and the resulting supernatant was precipitated by adjusting to 50% saturation with $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation at 27,000 g for 1 hr at 4°C and then resuspended in a minimal volume of 0.5 M phosphate buffer pH 6.1 containing 0.005 M EDTA. This material was dialysed against the same

buffer and then ultra-centrifuged at 90,000 g for 5 hrs at 4°C. The soluble supernatant contained both the Fc receptor activity and the phage associated lysin activity and was subjected to further purification.

Previously Fischetti et al. (1971) had defined conditions under which that the phage-associated lysin binds to cellulose phosphate. Using these conditions, 20 ml of the crude phage lysate was applied to a 1.5 x 16 cm cellulose phosphate (Whatman P11) column which was equilibrated with 0.1 M KH_2PO_4, pH 6.1, containing 0.005 M EDTA and 10% glycerol. Once the OD_{280} had returned to base line, the column was eluted with the same buffer containing 0.4 NaCl. In aggreement with the previous report (Fischetti et al., 1971), the phage-associated enzyme was eluted from the cellulose phosphate under these conditions (Fig. 4-1). Aliquots of the fractions collected tested for Fc receptor activity showed that 98% of the total recovered activity and 95% of the total recovered OD_{280} passed directly through the column.

The phage associated lysin can be stabilized and stored as described by Fischetti et al. (1971). The cellulose phosphate step does not result in any significant purification of the Fc receptor; however, I felt that the ability to isolate the crude phage associated lysin represented a significant biproduct of the purification.

In the next step of the purification procedure all fractions containing Fc receptor activity from the cellulose phosphate flow through material were dialyzed against 0.015 M NaCl and applied to a DEAE column equilibrated with 0.015 M NaCl, pH 7.4, and the unbound material was eluted in 0.015 M NaCl. Once the OD_{280} had returned to base line values, a linear gradient of NaCl from 0.05-0.5 M was

applied and finally, the column was eluted with 1.5 M NaCl. The NaCl

concentration was followed in the collected fractions by measuring

conductivity and the soluble FcRc activity was monitored using the

competitive binding assay described in Chapter 2. The majority of

the Fc receptor activity was recovered in a single peak (peak I) which

was eluted between a NaCl concentration of 0.12 and 0.18 M (see

Fig. 4-2). A second peak (peak II) containing approximately 5% of the

recovered activity was obtained at a NaCl concentration close to

0.24 M.

Fractions containing Fc receptor activity from the DEAE peak I

were pooled and concentrated by Amicon ultrafiltration using a PM10

(molecular weight cut off of 10,000) and further purified by applying

to a column of human IgG immobilized on Affi-gel 15. The column was

washed with 0.15 M PBS, pH 7.4 to remove unbound material and the bound

Fc receptors was eluted from the column using 0.1 M glycine-HCl,

pH 2.0. The eluted fractions were dialyzed against PBS, pH 7.4 and

tested for functional Fc receptor activity and protein content. The

resulting product contained 5,334 Fc receptor units/ml and 58 μg/ml of

protein. There was no detectable sugar as measured by the phenol

sulphuric acid method (Dubois et al., 1956). The overall purification

achieved by this procedure is summarized in Table 4-1. The affinity

purified Fc receptor was then tested for functional and chemical

homogeneity.

Functional Activity and Properties of the Isolated Fc Receptor

The affinity purified Fc receptor was concentrated 10 fold by

Amion Ultrafiltration using a PM-10 membrane. One hundred microliters

of this material, containing approximately 5×10^3 Fc receptor units
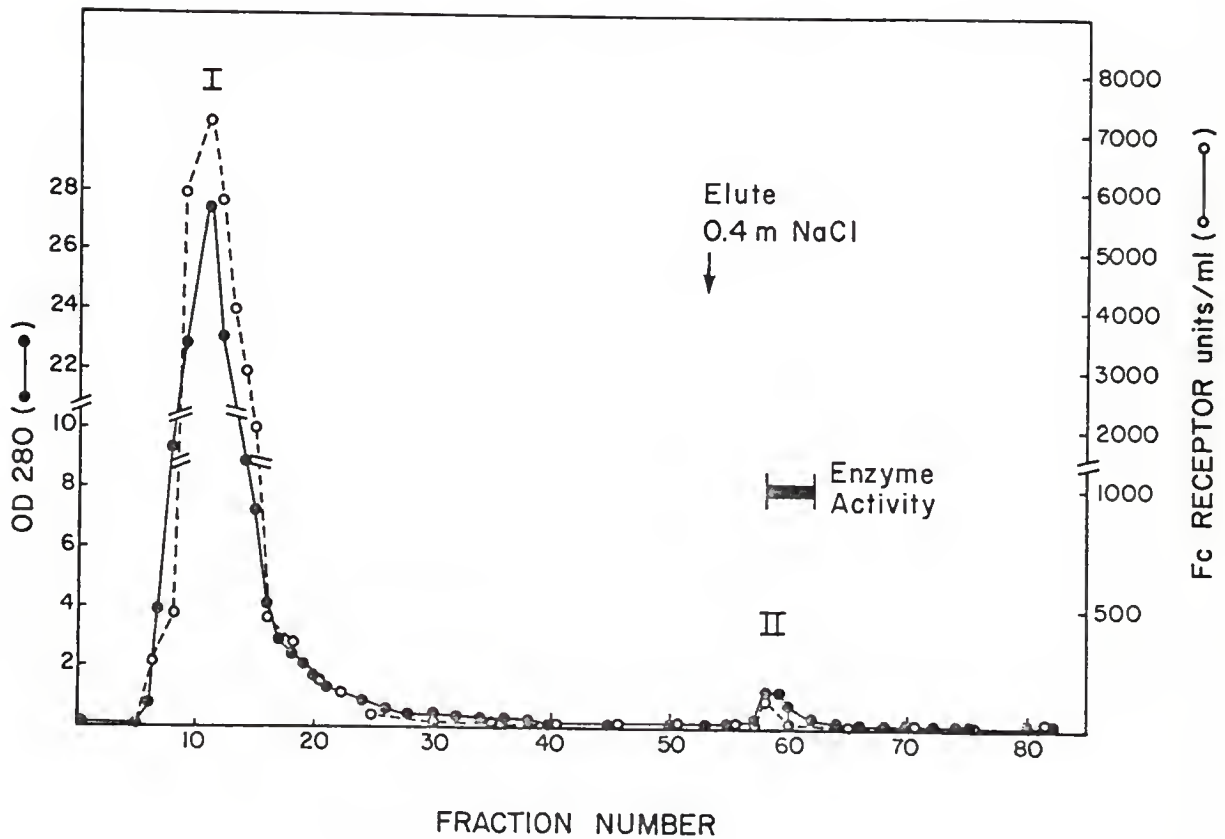
Figure 4-1. Cellulose phosphate chromatography of 50% $(NH_4)_2$
preparation of crude phage lysate. Twenty milliliters of
the crude phage lysate was applied to a 1.5 x 16 cm column
of cellulose phosphate pre-equilibrated in 0.1 M KH_2PO_4,
pH 6.1, containing 0.005 M EDTA and 10% glycerol. The
unbound material was eluted in the same buffer and the
phage lysin eluted in the same buffer containing 0.4 M
'NaCl. Four milliliter fractions were collected. Fc
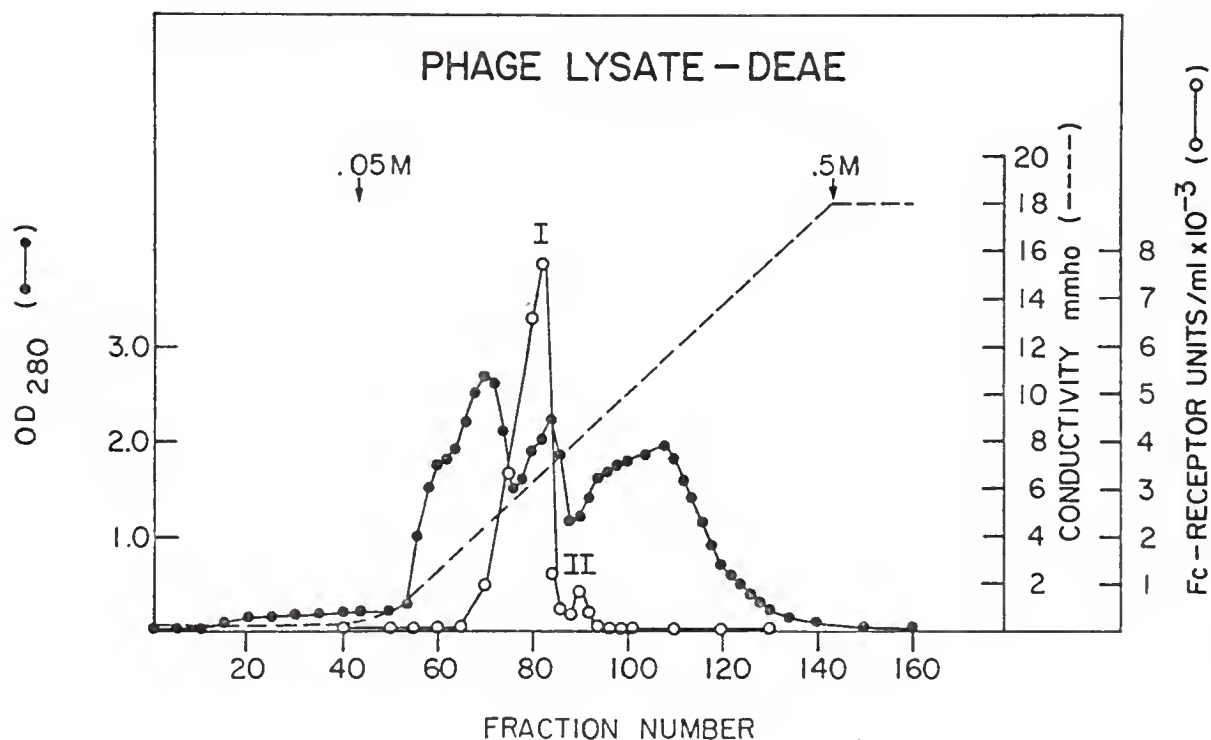receptor activity (o----o), OD_{280} (•——•).

Figure 4-2.   Ion exchange chromatography of phage lysate recovered from the pass through of a cellulose phosphate column. Fifty milliliters containing Fc receptor activity was applied to a 2.5 x 20 cm DEAE column, equilibrated in 0.015 M NaCl, pH 7.2 and eluted with a linear salt gradient from 0.05 M to 0.5 M. Five milliliter fractions were collected. Fc receptor activity (o——o), OD_{280} (●——●), conductivity (---).

TABLE 4-1

Partial Purification of a Group C Streptococcal Fc Receptor[a]

Fraction	Vol ml	FcRu /ml	FcRu Recovery Percent	OD_{280} /ml	OD_{280} Recovery Percent	Specific Activity $FcRu/OD_{280}$	Purifi-cation
Crude[b] Lysate	115	9,720	100	30.40	100.0	320	1.0
Cellulose Phosphate	560	1,302	65	4.30	69.0	302	0.9
DEAE	170	3,876	60	2.00	10.0	1,967	6.0
Immobilized IgG	66	5,334	32	0.10	0.2	53,340	167.0

a = One unit of Fc receptor activity (FcRu) is the concentration of material required to inhibit the binding of ^{125}I protein A by 30%.

b = The crude lysate refers to the cell free 50% ammonium sulfate cut as described in the Results.

and 58 µg of protein, was iodinated using the Immunobead reagent. When an aliquot of the iodinated, affinity-purified Fc receptor was mixed with immobilized human IgG, 96% of the radioactivity could be removed by two adsorptions. This adsorption was not inhibited by the addition of human IgG $F(ab')_2$ fragments derived from the same isolated IgG pool used to prepare the immobilized human IgG beads. These results demonstrated that the recovered affinity purified Fc receptor was functionally active and that binding was through the Fc region of IgG. Treatment of the labeled Fc receptor with trypsin resulted in a time dependent loss of binding to immobilized human IgG further indicating the protein nature of the receptor.

Fifty-eight micrograms of unlabeled affinity purified Fc receptor was applied to duplicate 7% non-denaturing polyacrylamide disc gels. One gel was stained with Coomassie blue while the second gel was sliced and eluted into 0.15 M VBS-gel pH 7.4 for 72 hrs. The functional activity in the eluted samples was measured using the competitive binding assay described in the Methods. The results presented in Figure 4-3B demonstrate that four major protein bands were detected by staining and these bands corresponded to the functional Fc receptor activity. A similar pattern was observed when radiolabeled Fc receptor was applied to gels and the distribution of ^{125}I monitored (Fig. 4-4). The distribution of counts indicated that band I contained 22%, band II (the major stained band) contained 26%, band III contained 11% and band IV contained 8% of the labeled Fc receptor material respectively. The remainder of the counts were dispersed at low levels throughout the gel (Fig. 4-4). The crude lysate electrophoresed under similar conditions demonstrated a similar pattern of functional
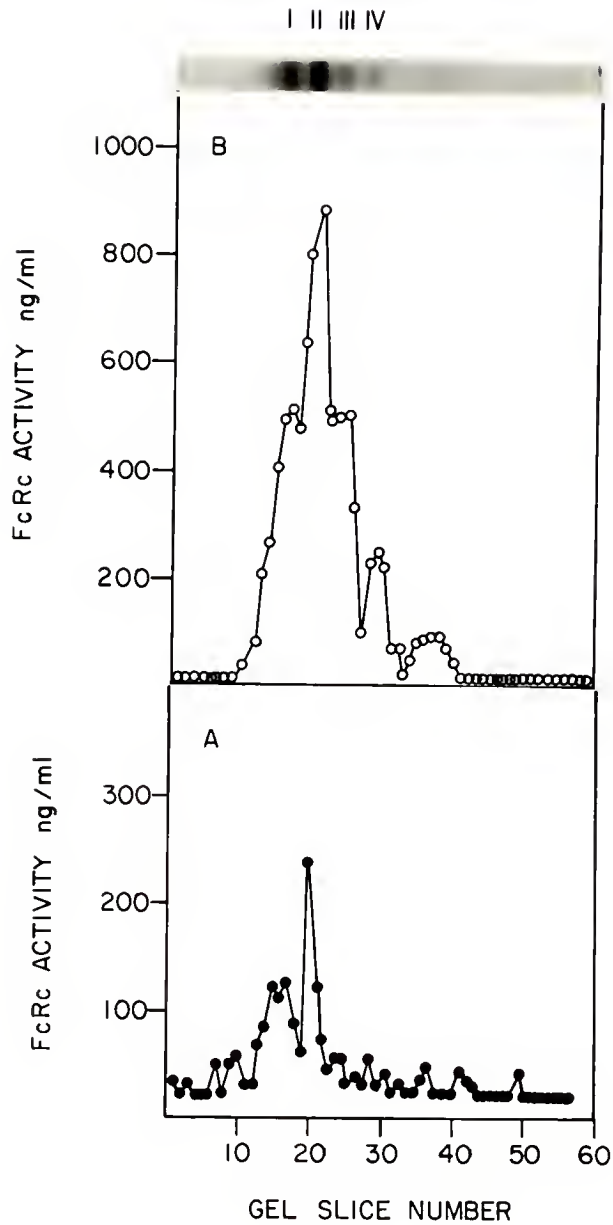
Figure 4-3. Nondenaturing polyacrylamide gel electrophoresis of
affinity purified Fc Rc and crude phage lysate containing
Fc Rc'.
A.   Functional Fc Rc activity in crude phage lysate
following electrophoresis and elution of gel slices into
VBS-gel for 72 hrs.
B.   Affinity purified Fc Rc, 30 µg, was applied to parallel
gels. The functional activity of eluted gel slices is
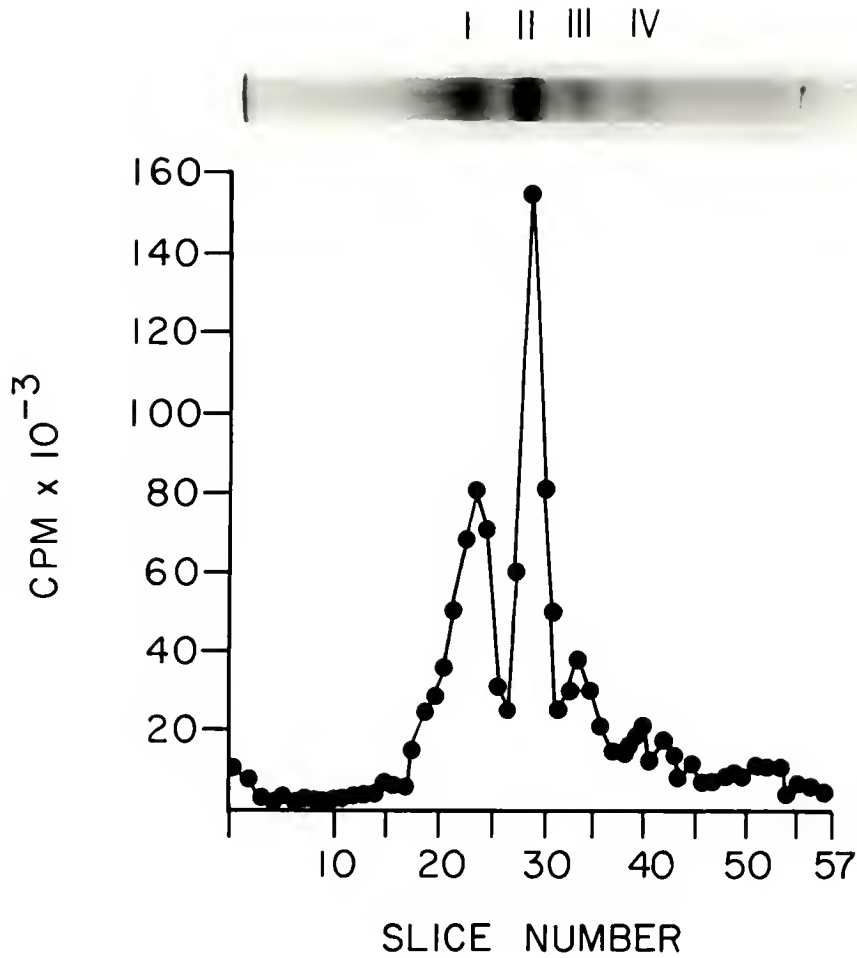compared with a gel stained with Coomassie blue.

Figure 4-4. Nondenaturing polyacrylamide gel electrophoresis of affinity purified unlabeled FcRc (30 µg) and ^{125}I-labeled FcRc (2×10^6 cpm).
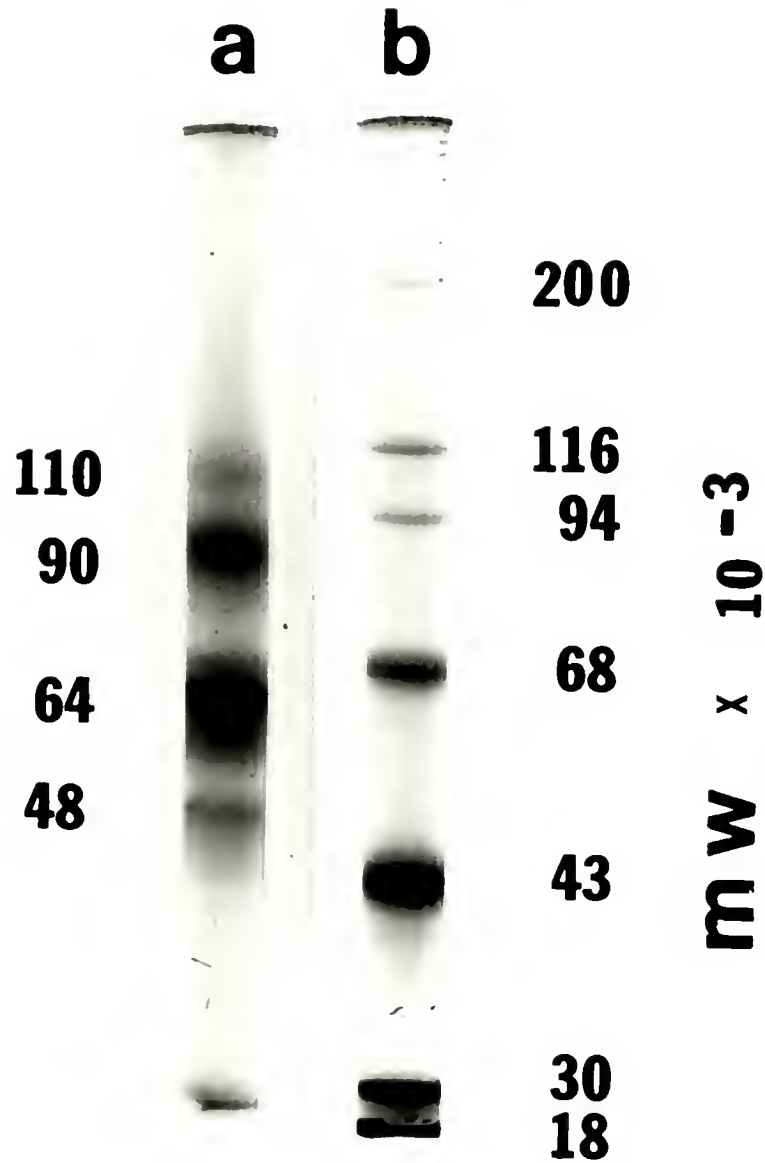
Figure 4-5. SDS polyacrylamide gel electrophoresis of 20 µg of
unlabeled affinity purified FcRc (a). Molecular weight
standards were included for reference (b).

activity indicating that the four peaks did not develop during the purification procedure (Fig. 4-3A).

Four major diffuse bands were also observed on SDS gels with molecular weights of 110,000, 90,000, 64,000, and 48,000 respectively. The predominant stained protein species was the 64,000 molecular weight protein (Fig. 4-5).

To determine whether the observed heterogeneity of Fc receptor activity represented distinct receptors or a common receptor with differing cell wall constituents covalently linked, two approaches were used. In the first, each of the active fractions of radiolabeled Fc receptor recovered by elution from nondenaturing polyacrylamide gels was tested for its ability to be inhibited from binding to immobilized human IgG by various concentrations of unfractionated unlabeled Fc receptor in the competitive binding assay. The results presented in Figure 4-6 demonstrated superimposable inhibition curves for each fraction and would suggest that the Fc receptor activity in each peak was directed against a similar site on the Fc region of the immobilized human IgG and that each receptor demonstrated a similar affinity.

The second approach to study the interrelationship of the four charged species of functionally active Fc receptor was to prepare antibody to the major Fc receptor activity. The affinity purified Fc receptor was separated by electrophoresis on a series of nondenaturing polyacrylamide gels. Each gel was stained and the region of the gel containing the major stained protein band was cut out, emulsified in complete Freund's adjuvant and injected into chickens following the immunization schedule detailed in the Methods. The production of antibody was followed by the ability of the immune chicken serum to
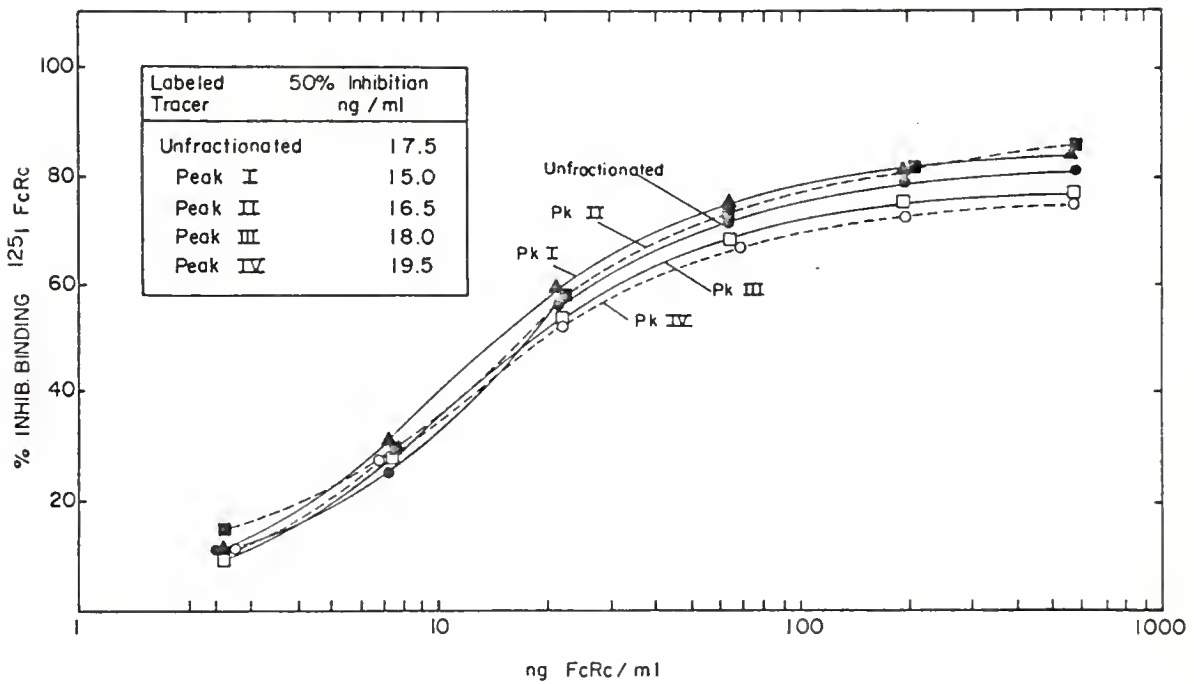
Figure 4-6. Inhibition of binding of ^{125}I-affinity purified FcRc
and its components to immobilized human IgG by unlabeled,
unfractionated FcRc. Individual peaks of ^{125}I-FcRc
correspond to the four major changes species eluted from
nondenaturing polyacrylamide gels. ^{125}I-FcRc,
unfractionated (o——o), and fractions eluted from
nondenaturing gels, ^{125}I-FcRc peak I (▲——▲),
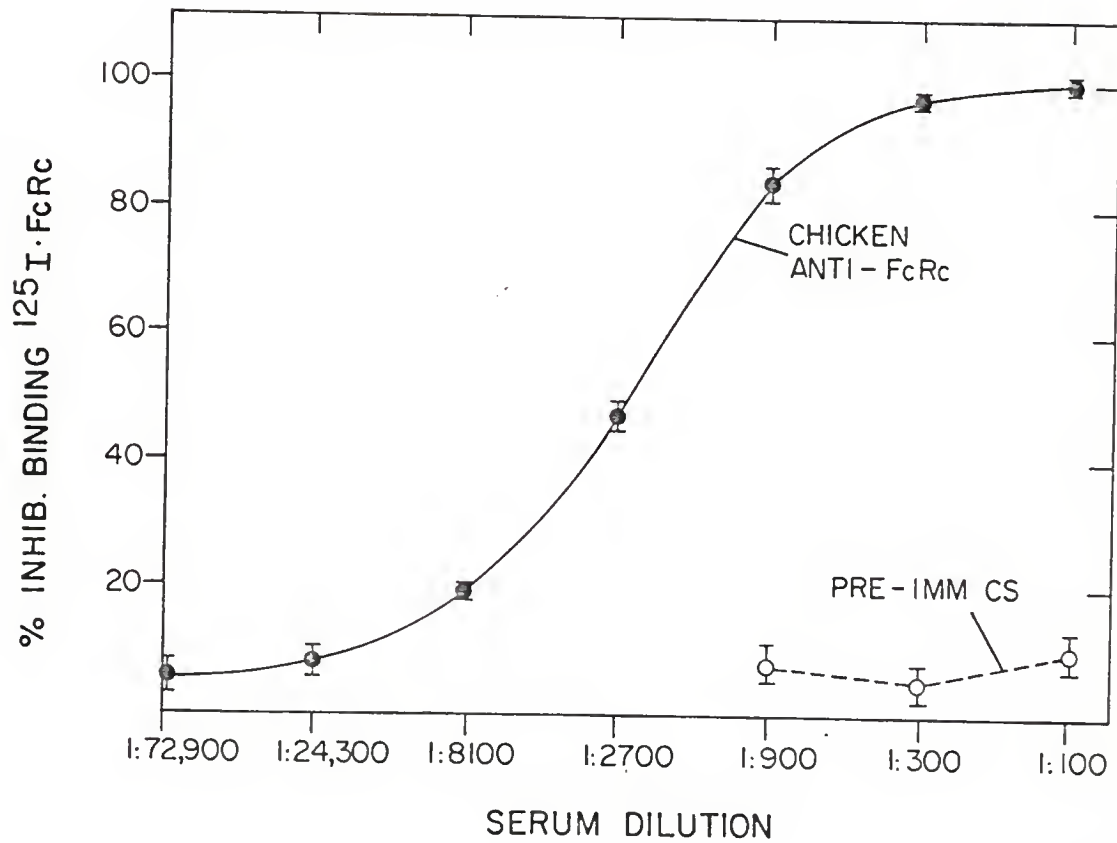[125]I-FcRc peak II (■---■), ^{125}I-FcRc peak III
(□——□), ^{125}I-FcRc peak IV (O---O).

Figure 4-7.   Inhibition of binding of affinity purified unfractionated
^{125}I-FcRc to immobilized human IgG by chicken antibody
prepared against the major charge species (peak II) in the
FcRc preparation. Chicken anti-FcRc (●——●), pre-immune
chicken serum (○----○).

inhibit binding of ^{125}I Fc receptor to immobilized human IgG beads.
The results presented in Figure 4-7 demonstrate that the resulting
antibody could completely inhibit binding of the ^{125}I labeled
unfractionated Fc receptor to immobilized human IgG. Chicken serum
obtained prior to immunization was without effect. The labeled tracer
contains all four major charge species of Fc receptor (see Fig. 4-4)
and the antibody was prepared only against the second peak which
contains 26% of the total Fc receptor activity. These findings suggest
that each of the four peaks in the affinity purified Fc receptor
preparation contains antigenically related structures. Taken together
the results in Figures 4-6 and 4-7 would suggest that the group C
streptococcus has a single functional Fc receptor that is extracted
with differing covalently bound fragments that account for the
heterogeneity observed on nondenaturing and SDS polyacrylamide gels.

Attempts to establish conditions to convert the four peaks to a
single functionally active molecular weight form by treatment with a
variety of enzymes have not been successful.

Discussion

In this chapter the purification and partial characterization of a
group C streptococcal Fc receptor is described. A number of extraction
procedures were tested including phage lysis, hot acid and alkali
extraction and treatment with a variety of enzymes including pepsin,
lysostaphin, lysozyme and mutanolysin. Soluble Fc receptor activity
was observed following hot acid extraction, phage lysis or treatment of
the group C streptococcus with mutanolysin. The most favorable
starting material for further purification was found to result from

phage lysis. This extraction procedure resulted in the highest yield
of soluble Fc receptor activity with the least amount of charge and
size heterogeneity.

The Fc receptor solubilized following phage lysis was stable for
at least one month at 4°C and for a minumum of six months at -70°C and
at no time was protease activity detectable in any extract from this
bacteria. The Fc receptor activity was stable to hot acid, destroyed
by hot alkali and destroyed by trypsin.

The Fc receptor activity could be isolated to functional
homogeneity by sequential $(NH_4)_2SO_4$ precipitation, cellulose
phosphate chromatography, DEAE ion exchange chromatography and by
binding to and selective elution from a column of immobilized human
IgG. [The cellulose phosphate step is not essential and similar
results were obtained when this step is omitted. The advantage of
including this step is that the phage associated lysin can be recovered
in a reasonably pure form and can be further purified as described
previously by Fischetti et al. (1971).]

All of the material recovered from the immobilized IgG column was
functionally active as judged by its ability, following radioiodina-
tion, to bind to immobilized IgG. This binding was unaffected by the
presence of $F(ab')_2$ fragments, indicating the receptor was binding
a site on the Fc region of human IgG. The functionally active Fc
receptor was physicochemically heterogeneous being resolved into four
major bands on non-denaturing polyacrylamide gels. A similar pattern
with four major diffuse bands was also observed on SDS gels. The major
protein bands had molecular weights of 110,000, 90,000, 64,000 and
48,000 daltons.

Despite the obvious heterogeneity in the size and charge of the solubilized Fc receptor it appeared to demonstrate remarkable uniformity in its binding to the Fc region of human IgG. Similarly, when the cold unfractionated material was used to compete with individual labeled peaks eluted from gels, superimposable inhibition curves were observed with all combinations (see Fig. 4-6). Similarly, when each of the individual unlabeled peaks eluted from non-denaturing polyacrylamide gels was tested for its ability to compete with the radiolabeled unfractionated receptor, superimposable inhibition curves were also observed (Chapter 5). These results suggested that for all practical purposes the affinity purified Fc receptor preparation contained a single functional activity, i.e., it bound to the same site on the Fc region of IgG with a constant affinity. An antibody prepared against the major charge species of the solubilized Fc receptor preparation was found to be capable of totally inhibiting the functional activity of the unfractionated Fc receptor (see Fig. 4-7). These results would suggest that the size heterogeneity and apparent functional homogeneity most probably results from the solubilization of a single receptor molecule covalently linked to various other cell wall constituents.

Heterogeneity of this type has been observed in earlier studies attempting to isolate the M protein from streptococcal cell walls (Fox and Wittner, 1969; Fischetti et al., 1976; Kühnemund et al., 1981). More recently, extraction conditions have been established that allow a single minimal molecular weight form of the M protein to be isolated (Manjula and Fischetti, 1980).

In the initial attempts to convert the heterogeneous soluble Fc receptor preparation to a single species I have tested pepsin, trypsin

and lysozyme treatments under a variety of optimal and suboptimal conditions for enzyme action. To date, none of these treatments has been successful in reducing the number of protein bands, and pepsin and trypsin both lead to a dose-dependent loss of functional activity.

The Fc receptor isolated here was recovered in a higher yield than previously reported. I am able to recover 400 μg of affinity purified FcRc/g wet weight bacteria extracted, compared to the maximum yield previously reported of 10 μg/g wet weight of bacteria extracted (Grubb et al., 1982). The heterogeneity observed was similar to that described by others. The most homogeneous form of a streptococcal Fc receptor reported was the one isolated by Grubb et al. (1982) that resulted from alkaline extraction of a group A streptococcus. This receptor was isolated in a predominant 29,500 molecular weight form only when protease inhibitors were present. This receptor differed markedly from the receptors described here. The smallest of these group C Fc receptors was 48,000 daltons and the functional activity was totally destroyed by treatment with hot alkali, the condition used by Grubb et al. (1982) for their initial extraction. In addition, with the group C streptococcus used here there was no evidence of protease contamination, degradation or change in heterogeneity of my soluble Fc receptor during purification (Fig. 4-3).

The ability to isolate, in high yield, a functionally active streptococcal Fc receptor with apparent homogeneity in binding to the Fc region of IgG represents a potentially useful immunochemical reagent. Staphylococcal protein A, by virtue of its selective Fc binding activity, has proved to be extremely valuable when radio or enzyme labeled as a tracer in immunoassays (Langone, 1978, 1982b; Gee

and Langone, 1981), or once immobilized for isolation of various
classes and subclasses of IgG (Ey et al., 1978; Patrick and Virella,
1978), separation of antigen-antibody complexes (Kessler, 1976;
MacSween and Eastwood, 1978) or for selective removal of IgG from serum
(Boyle and Langone, 1980; Langone et al., 1979a; Goding, 1978). If the
Fc receptor isolated from this group C streptococcus has species or
subclass reactivities different from staphylococcal protein A it should
be valuable for expanding the immunochemical approaches currently using
protein A. A comparison of the reactivity of staphylococcal protein A
and the streptococcal Fc receptor is presented in the following
chapter.

CHAPTER FIVE
COMPARISON OF THE FUNCTIONAL AND ANTIGENIC
RELATIONSHIP OF A GROUP C STREPTOCOCCAL Fc RECEPTOR
WITH STAPHYLOCOCCAL PROTEIN A

Introduction

Studies of bacterial Fc receptors on streptococci by Myhre and Kronvall (1981b) have suggested that there are five bacterial Fc receptors with differing ranges of species and subclass reactivities. These receptors, if they could be obtained in a solubilized functionally homogeneous form might then be anticipated to extend the usefulness of bacterial Fc receptors beyond those already described for protein A. In the preceding chapter I described a method for the isolation of a functionally homogeneous Fc receptor from a group C streptococcus and in this chapter I will compare the functional activities of this receptor to those of staphylococcal protein A. The results presented suggest that this isolated streptococcal Fc receptor (FcRc) has the binding characteristics of the type III receptor described by Myhre and Kronvall (1981b) based on the Fc-reactivities of heat-killed streptococci.

Materials and Methods

Purified Streptococcal Fc Receptor (FcRc)

The soluble streptococcal Fc receptor (FcRc) was isolated and purified to functional homogeneity from a group C strain designated 26RP66 as described in Chapter 4.

Polyacrylamide Gel Electrophoresis

The affinity purified FcRc was separated by electrophoresis into four functionally active fractions on 7% non-denaturing polyacrylamide gels as described in Chapter 4.

Iodination of PA and FcRc

Purified protein A (Pharmacia) and the affinity purified FcRc were iodinated by the mild lactoperoxidase method using enzyme beads (Bio-Rad) as described previously Chapter 2.

Competitive Binding Assay for Functional PA and FcRc Activity

Protein A and FcRc were quantified using a modification of the competitive binding assay of Langone et al. (1977). In this assay 0.2 ml of a test sample or buffer is mixed with 0.2 ml of a standard suspension of agarose beads with covalently coupled human, rabbit or goat IgG (Bio-Rad Laboratories, Richmond, California), and 0.1 ml of ^{125}I protein A or ^{125}I FcRc (approximately 20,000 cpm) and incubated at 37°C for 90 mins. Two milliliters of EDTA-gel was added to each tube and centrifuged at 1,000 g for 5 mins and the supernatant fluid decanted. After an additional wash, the radioactivity associated with the beads was determined in an LKB Gamma Counter. The number of counts bound in the absence of fluid phase PA or FcRc was compared to the number of counts bound to the beads in the presence of known amounts of fluid phase PA or FcRc and the degree of inhibition determined. The functional activity of these two receptors was compared by competing unlabeled PA or FcRc with either ^{125}I-PA or ^{125}I-FcRc.

Immobilized Human, Rabbit and Goat IgG

Human IgG was coupled to Immunobeads (Bio-Rad) for use in the competitive binding assays as described by Langone et al., 1979a. Rabbit and goat IgG covalently coupled to Immunobeads (Immunobead R-1 and Immunobead G-1, respectively) were obtained from Bio-Rad, Richmond, California.

Chicken Antibodies to Protein A and FcRc

Monospecific antiserum to staphylococcal protein A was a gift from Dr. John Langone, National Cancer Institute, Bethesda, Maryland. The antiserum was prepared as described in Chapter 3. Monospecific anti-serum to streptococcal FcRc was prepared as described in Chapter 4.

Competitive Binding Assay for the Quantitation of IgG

IgG from a variety of species was quantitated by a competitive binding assay developed by Langone et al. (1977) and modified as described in Chapter 2. For this study the ability of different species IgGs to inhibit the binding of either ^{125}I-PA or ^{125}I-FcRc to immobilized human IgG was compared.

Immunoglobulins

Stock human IgG was prepared by chromatography of normal human serum on DEAE cellulose (Boyle and Langone, 1980). Aliquots were stored at -70°C until use. Purified rabbit, cow, sheep, goat, rat, dog, and pig IgG were purchased from Cappel (Cappel Laboratories, Inc., Cochranville, PA).

Human IgG Subclasses

Human IgG subclasses were provided by the WHO/IUIS Immunoglobulin Subcommittee. Two samples of each subclass were tested:

IgG_1 (k) lot #0781 and IgG_1 (λ) lot #0180

IgG_2 (k) lot #0380 and IgG_2 (λ) lot #0981

IgG$_3$ (k) lot #0282 and IgG$_3$ (λ) lot #0381

IgG$_4$ (k) lot #0981 and IgG$_4$ (λ) lot #0880

Results

Inhibition of Binding of ^{125}I PA or ^{125}I FcRc to Immobilized Human IgG by Unlabeled PA or FcRc

The isolated functionally active FcRc has previously been demon-
strated to be composed of four major charged species, that can be
readily separated and recovered following electrophoresis and elution
from non-denaturing polyacrylamide gels (Chapter 4). Each fraction
eluted from the gel has been shown to bind the Fc region of IgG and all
are antigenically related (Chapter 4).

In the initial experiments described here I compare inhibition of
binding of labeled tracer to immobilized human IgG by 1) unlabeled
protein A, 2) unlabeled affinity purified FcRc or, 3) affinity purified
FcRc that was further fractionated on polyacrylamide gels. The results
presented in Figure 5-1A demonstrate that binding of ^{125}I PA could
be inhibited by any of the FcRc fractions tested and each FcRc fraction
showed a superimposable inhibition curve. These results indicated that
the binding site on the Fc region of human IgG for protein A and FcRc
are either identical or in close proximity. When the experiment was
repeated using ^{125}I FcRc as tracer similar results were obtained
(see Fig. 5-1B). As expected the FcRc was more effective in inhibiting
binding of ^{125}I FcRc to the immobilized human IgG than in
inhibiting ^{125}I PA. By contrast, protein A demonstrates equivalent
inhibition with both tracers, suggesting that its affinity for the Fc
region of human IgG is higher than that of the FcRc. In similar
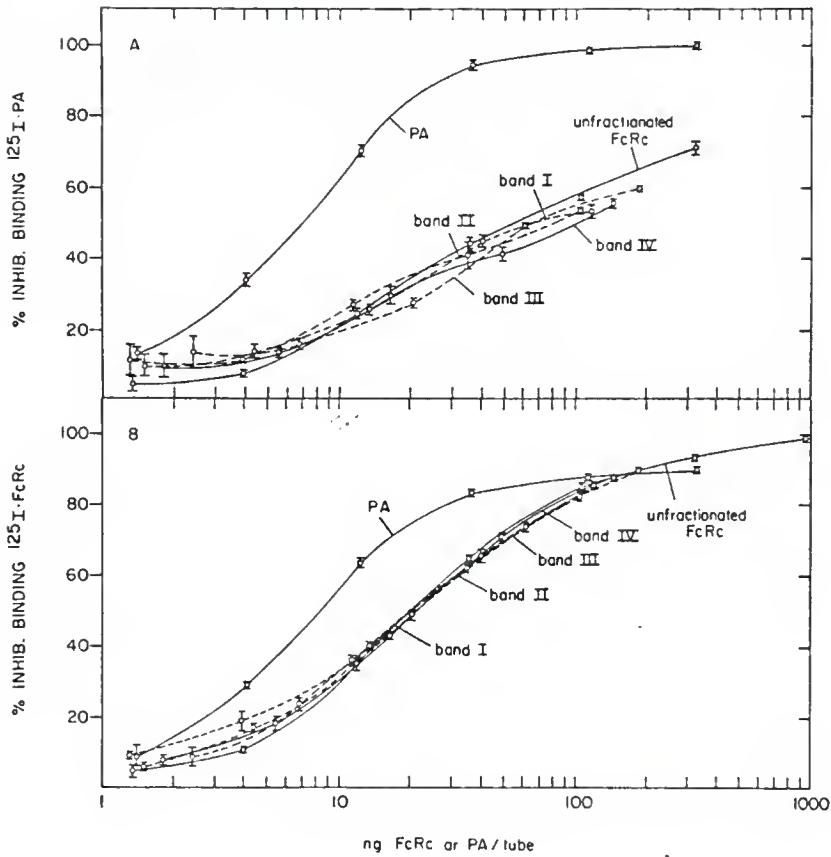comparative binding assays using immobilized rabbit or goat IgG

Figure 5-1.   Inhibition of binding of (A) ^{125}I-PA or (B)
              ^{125}I-FcRc to immobilized human IgG by unlabeled PA,
              affinity purified unfractionated FcRc or the major FcRc
              charge species. For precise experimental details, see
              text.

in place of human IgG, no heterogeneity in binding was observed within any of the affinity purified FcRc fractions. These findings would support my previous conclusion that the charge and size heterogeneity of the affinity purified FcRc preparation could be attributed to covalently linked cell wall constituents attached to a single type of receptor (Chapter 4). Consequently in the remaining experiments presented here I have compared the activity of the total FcRc preparation to protein A.

Antigenic Relationship of Protein A and FcRc

Polyclonal antibodies to protein A or to the major charge species of FcRc were prepared in chickens as described in Chapters 3 and 4. Each antibody was tested for its ability to prevent binding of labeled ^{125}I PA or ^{125}I FcRc to immobilized human IgG. In this assay labeled tracer and immobilized human IgG were incubated for one hour at 37°C with dilutions of serum containing antibody to protein A, serum containing antibody to FcRc or normal chicken serum. The quantity of radiolabel associated with the immobilized IgG was quantified after washing to remove soluble antigen-antibody complexes containing the labeled tracer. Inhibition detected in this assay requires that the antibody will combine with a site on the Fc receptor that will sterically inhibit its interaction with the corresponding binding site on IgG. The results presented in Figure 5-2 indicated that the binding of protein A or FcRc was only inhibited when the corresponding antibody was used. There was no evidence of any antigenic crossreactivity between these two bacterial Fc receptors. Neither protein A nor the FcRc reacted with any component in normal chicken serum.
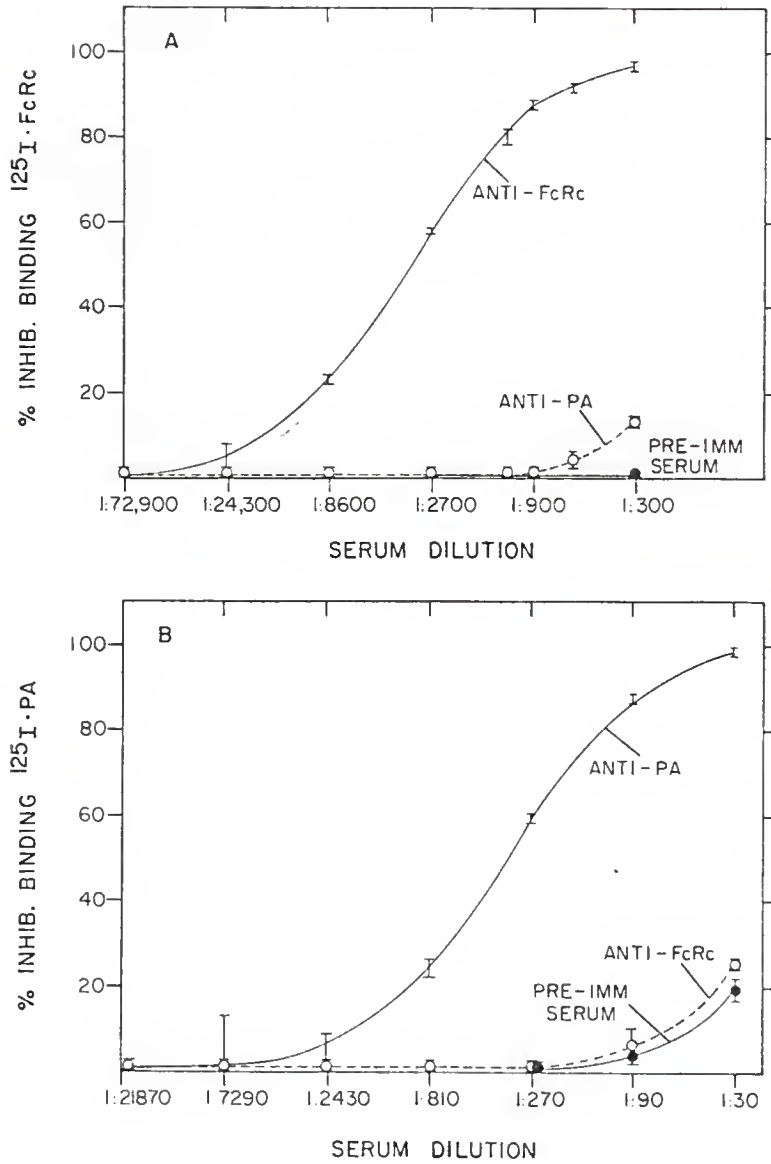
Figure 5-2.   Inhibition of binding of (A) ^{125}I-FcRc or (B)
^{125}I-PA to immobilized human IgG by antibody against
the major charge species of FcRc or against PA. (A)
anti-FcRc (——), anti-PA (O---O), pre-immune chicken
serum (●——●); (B) anti-PA (——), anti-FcRc (o---o),
pre-immune chicken serum (●——●).

Comparison of Species Reactivity of Protein A and FcRc

Using the competitive binding assay described in the Methods the ability of different species of IgG to inhibit binding of ^{125}I PA or ^{125}I FcRc to immobilized human IgG were compared. The results presented in Figure 5-3 and Table 5-1 demonstrate a number of clear differences in binding of the two Fc receptors. In particular, sheep, cow and goat IgG were much more reactive with the FcRc than with protein A (Figure 5-3). Under the assay conditions used similar inhibition was observed using rabbit IgG, however protein A was more efficient in its reactivity with human IgG than the FcRc. An absolute comparison of reactivities of protein A and FcRc can not be made since the FcRc preparation is heterogeneous and accurate estimates of the specific activity of the ^{125}I labeled tracer cannot be made.

The reactivities of human IgG subclasses were also compared in similar experiments. The results presented in Figure 5-4 demonstrate a number of interesting reactivities. The labeled FcRc reacted with all four human subclasses with IgG_3 and IgG_1 showing approximately equivalent reactivity while IgG_2 and IgG_4 demonstrated lower reactivity. There was considerable variability between the two myeloma proteins of each subclass tested. It is not clear whether these differences relate to unique receptors on immunoglobulins from different individuals, e.g., allotypic sites (Haake et al., 1982; Schalén, 1982), reactions with Fab regions (Inganäs, 1981; Erntell et al., 1982) or differences in amino acid composition of myeloma proteins within the site where the bacterial receptor binds. Examples of each of these types of reactivity of bacterial Fc receptors have been
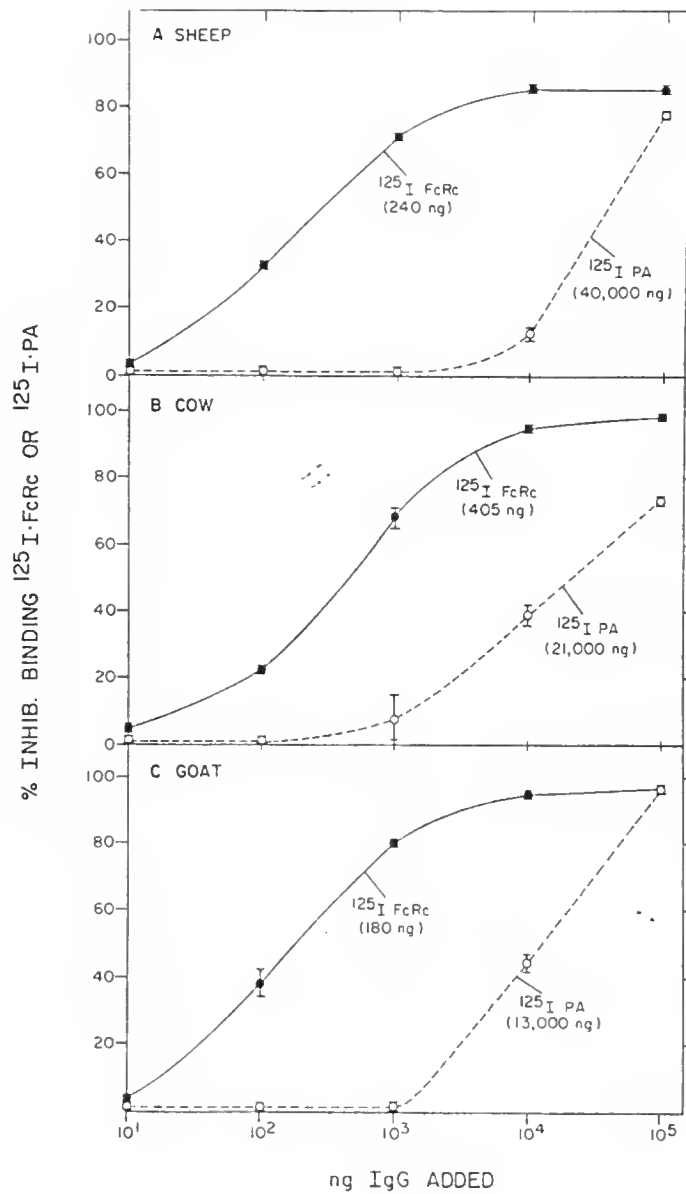
Figure 5-3. Inhibition of binding of ^{125}I-FcRc or ^{125}I-PA to
immobilized human IgG by sheep (A), cow (B), or goat (C)
IgG. ^{125}I-FcRc (●——●), ^{125}I-PA (o---o); numbers
in parenthesis represent the concentration of IgG required
to inhibit by 50% the binding of the labeled tracer to
immobilized human IgG. For precise experimental details,
see text.

TABLE 5-1

Inhibition of Binding of ^{125}I-PA or ^{125}I-FcRc to
Immobilized Human IgG by IgG from Different Species

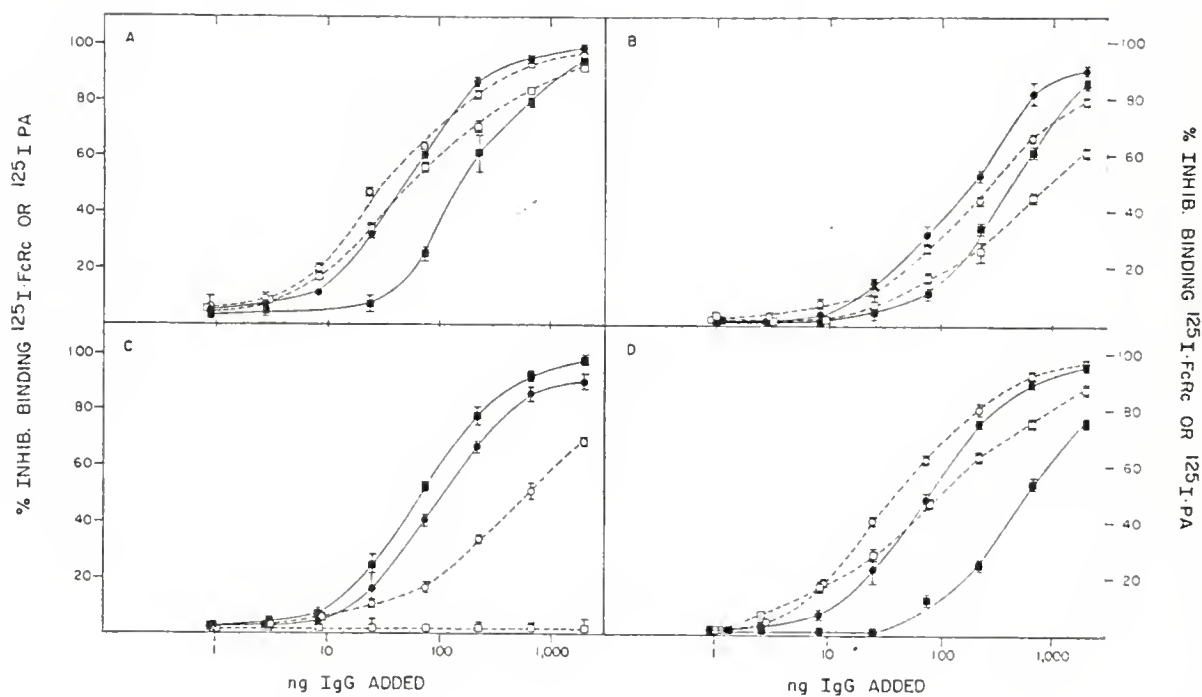| Species	Nanograms IgG Required to Inhibit by 50%	
	^{125}I-FcRc	^{125}I-PA
Rabbit	125	130
Human	44	13
Pig	70	118
Goat	180	13,000
Sheep	240	40,000
Cow	405	21,000
Dog	13,000	100
Rat	$>10^5$	$>10^5$

Figure 5-4.   Inhibition of binding of ^{125}I-PA or ^{125}I-FcRc to
                immobilized human IgG by human IgG subclass standards
                IgG_1 (A), IgG_2 (B), IgG_3 (C), or IgG_4 (D).
                Reactivity of individual subclass standards, k light
                chains, with ^{125}I-FcRc (●——●); reactivity of
                individual subclass standards, k light chains, with
                ^{125}I-PA (○---○); reactivity of individual subclass
                standards, λ light chains, with ^{125}I-FcRc (■——■); and
                reactivity of individual subclass standards, λ light
                chains, with ^{125}I-PA (□---□).

TABLE 5-2

Inhibition of Binding of ^{125}I-PA or ^{125}I-FcRc to
Immobilized Human IgG by Human Myelomas

IgG Subclass	Nanograms IgG Required to Inhibit by 50%	
	^{125}I-FcRc	^{125}I-PA
IgG$_1$ (k)	50	30
IgG$_1$ (λ)	165	60
IgG$_2$ (k)	190	265
IgG$_2$ (λ)	465	960
IgG$_3$ (k)	120	666
IgG$_3$ (λ)	70	none detected
IgG$_4$ (k)	80	39
IgG$_4$ (λ)	580	90

described (Inganäs, 1981; Haake et al., 1982; Schalén, 1982; Erntell et al., 1983; Shimizu et al., 1983).

Protein A reacted most efficiently with IgG_1 and IgG_4 and with a lower efficiency with IgG_2. The reactivity of two purified IgG_3 preparations isolated from myeloma serum showed two distinct reactivities. One IgG_3 preparation failed to react with protein A ($IgG_3 \lambda$ #0381), while a second preparation of IgG_3 (IgG_3 k #0282) showed low but significant reactivity with protein A. These types of differences in IgG_3 reactivities with protein A have previously been reported and can be related to the allotype of the immunoglobulin molecule (Haake et al., 1982). As noted above, a considerable difference in reactivity of the two myeloma proteins of a given subclass was also observed in the reactivity towards protein A, Table 5-2.

Discussion

Five distinct bacterial Fc receptors have been described based on the functional reactivity of intact bacteria with different species and subclasses of IgG (Myhre and Kronvall, 1981b). With the exception of staphylococcal protein A, the type I receptor, attempts to isolate a functional Fc receptor which retains the binding characteristics observed on the bacterial surface have met with limited success. The previous chapter describes the isolation of an Fc receptor from a group C streptococcus that was recovered following solubilization of the cell wall by phage lysis, ion exchange chromatography, and binding to and elution from immobilized human IgG. This affinity purified strepto-coccal Fc receptor has been designated FcRc. The isolated FcRc was

functionally homogeneous and could be shown to bind selectively to the Fc region of human IgG. Despite the uniform functional activity of the FcRc preparation it was physicochemically heterogeneous being composed of four major charge and size species. In this chapter I have compared the reactivity of staphylococcal protein A to the total FcRc and to each of its four major constituents. In all of these studies I was unable to distinguish differences in reactivity among any of the four major peaks of FcRc and the unfractionated affinity purified material (see Fig. 5-1 and Chapter 4). Consequently, I have concluded that for practical purposes the affinity purified FcRc behaved functionally as a single species.

In competitive binding studies using $125I$ protein A as the tracer, unlabeled FcRc or unlabeled protein A could inhibit the binding of the labeled tracer. The shape of the inhibition curves indicated that protein A was a more efficient inhibitor. When the experiments were repeated using $125I$ FcRc as tracer, inhibition by unlabeled FcRc was most efficient, while protein A demonstrated similar inhibition against either labeled tracer. It is not possible to make any absolute comparison between the affinity of protein A and FcRc for human IgG because of the size heterogeneity in the affinity purified FcRc preparation and the resulting uncertainty in determining specific activity of the $125I$ FcRc tracer molecules. The competitive binding studies shown in Figure 5-1 do indicate, however, that the protein A and FcRc bind to either the same site or two distinct sites in close proximity on the Fc region of human IgG.

Despite this similarity in functional activity, protein A and the FcRc demonstrated no common antigenic determinants (Fig. 5-2).

Furthermore, when the reactivities of the two bacterial Fc receptors were tested against a variety of different mammalian IgG preparations a number of marked differences were observed. The FcRc was found to bind much more efficiently than protein A to sheep, cow, and goat immuno-globulins. The reactivity with pig, human or rabbit IgG did not differ by more than 4-fold under the assay conditions used. Differences in reactivity with human subclasses were also observed. The reactivity of protein A with human IgG sublcasses revealed an interesting pattern (Fig. 5-4). In particular, IgG$_3$ isolated from two patients with myeloma gave markedly different results. One sample, an IgG$_3$ λ, failed to react with protein A at all while the second sample, an IgG$_3$k, inhibited binding of protein A to immobilized IgG with 50% inhibition being acheived on addition of 666 ng. Differences in protein A binding to IgG$_3$ have previously been observed and have been attributed to an allotypic site present on IgG$_3$ (Haake et al., 1982; Shimizu et al., 1983). It has been shown that the replacement of an arginine by a histidine at residue 435 of the heavy chain in IgG$_3$ generates a site capable of binding protein A (Shimizu et al., 1983). My results indicate that protein A bound IgG$_1$ better than IgG$_2$ and IgG$_4$, see Figure 5-4. The FcRc was shown to bind to all four subclasses with IgG$_1$ and IgG$_3$ being the most reactive followed by IgG$_4$ and IgG$_2$. Considerable differences were observed between isolated human subclasses from different myeloma sera with either tracer. These differences may reflect non-Fc binding activities of the receptor or may be attributed to differences in the purity of the subclass reagents.

The functional reactivities of the soluble FcRc described here corresponds to those described by Myhre and Kronvall (1981b) as a type III receptor based on their studies using intact bacteria. The ability to isolate a streptococcal type III Fc receptor which maintains its functional activity has a number of clear practical implications. First, the radiolabeled or enzyme labeled FcRc can be used as a tracer in a variety of immunoassays. This approach has been used very successfully with protein A (Langone, 1982b) and has only been limited by the range of IgG species, isotypes and subclasses with which protein A reacts. As demonstrated in this report, the isolated FcRc has an extended range of reactivities beyond those of protein A. In particular, in contrast to protein A, FcRc reacts well with cow, goat and sheep immunoglobulins. Furthermore, the differences in reactivity of protein A and FcRc with human IgG subclasses suggest that these immobilized bacterial receptors either alone or in concert will be useful for affinity purification of these IgG subclasses. The implications of these findings are discussed in the next chapter.

CHAPTER SIX
CONCLUSION

Myhre and Kronvall have carried out extensive studies on the
distribution and functional characteristics of Fc receptors on
bacterial cell surfaces (see Chapter 1). Based on the functional
reactivities with different IgG classes and subclasses five types of Fc
receptors have been identified (Myhre and Kronvall, 1981b). With the
exception of staphylococcal protein A, the type I receptor, little
information is available on the structural and functional properties of
the other four types of receptors. Grubb et al. (1982) reported the
isolation of a homogeneous Fc receptor from a group A streptococcus
that was most probably a type II receptor, but they did not
characterize its functional activities. This receptor was recovered in
low yield (10 µg/g wet weight of bacteria) and was only isolated when
protease inhibitors were present.

In this study I have described the first isolation to functional
homogeneity of a soluble type III receptor. This has been achieved by
developing methods to detect bacteria rich in surface Fc receptors and
by using a competitive binding radioimmunoassay to follow the Fc
receptor activity once solubilized. The type III receptor I have
isolated was obtained in high yield, 400 µg/g wet weight of bacteria.
The soluble receptor was physicochemically heterogeneous being composed
of four major charged and sized species of 110,000, 90,000, 64,000, and

95

48,000 daltons. All forms of the Fc receptors displayed similar functional properties and shared common antigenic determinants.

A comparison of this type III receptor (FcRc) with staphylococcal protein A showed that they compete with one another for the same site, or a site in close proximity, on the Fc binding region of IgG. For protein A the histidine at residue 435 of the CH_3 domain has been shown to be critical (Haake et al., 1982; Shimizu et al., 1983). These two receptors have a different range of species reactivities. In particular the FcRc binds cow, sheep and goat IgG and the human IgG_3 subclass more efficiently than protein A.

The isolation to functional homogeneity of an Fc receptor that is distinct from protein A has a number of practical implications: 1) Radiolabeled or enzyme labeled protein A has already been demonstrated to be a very useful tracer in immunoassays (for review see Langone, 1982b). Its major limitation has been the range of IgG species with which it will react. The FcRc described here allows these approaches to be extended to use with antibodies prepared in sheep and goats. These species have been shown to be unsuitable for direct assays in which protein A is the tracer (Langone, 1980a). 2) Immobilized FcRc may be useful for separating or depleting IgG from serum or secretions, particularly for species with which the FcRc binds well that react poorly with protein A. The two immobilized bacterial receptors in conjunction may be useful for separating IgG subclasses in which significant differences in binding affinity exists, e.g., human IgG_3. 3) The biological function(s) of a type III streptococcal Fc receptor, in particular the complexes formed between FcRc and IgG and the role they may play in pathogenesis, can now be addressed. 4) The

availability of an antibody directed against a type III receptor will allow the distribution of this marker on various streptococci to be measured and its potential role as a pathogenic factor to be explored. 5) The availability of antibodies to type I and type III receptors should allow the approaches outlined in Chapter 3 to be extended to enable bacteria with other distinct Fc receptors to be identified and studied.

The methods described in this dissertation for detecting and solubilizing Fc receptors in general, and the type III receptor in particular, offer a number of new directions that can be explored in future studies.

First, methods of obtaining a minimum size receptor with functional activity and chemical homogeneity may be useful for future immunochemical studies. This might be achieved by converting the material obtained by phage lysis into a single minimal molecular weight species or by establishing a different extraction procedure that results in a more physicochemically homogeneous product. This would be particularly valuable for further documenting the properties of the FcRc enabling absolute measures of affinity, amino acid composition and primary sequence to be determined. Second, the immunoglobulin species, class and subclass reactivities can be further defined and the potential of the FcRc as an immunochemical tool explored. For example, the labeled purified receptor could be used in immunoassays using goat or sheep antibodies and immobilized purified FcRc or the killed bacteria could be used for immunoglobulin class and subclass separations. Third, antiserum to protein A or the FcRc could be used as described in this study to examine the antigenic relationship of other Fc receptor

types described by Myhre and Kronvall (1981b). Fourth, the extraction
and purification methods described here could be used to isolate Fc
receptors of types that are antigenically and possibly functionally
distinct. Finally, a number of biological functions can now be
examined, including complement activation, mitogenesis and the nature
of FcRc-IgG complexes. Additionally, the role these receptors may play
in virulence can be explored using the purified receptors in both in
vivo and in vitro systems.

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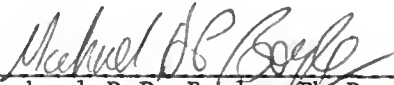
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BIOGRAPHICAL SKETCH

Kathleen June Reis was born on May 16, 1952, the middle child of
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was admitted to graduate school in the Department of Immunology and
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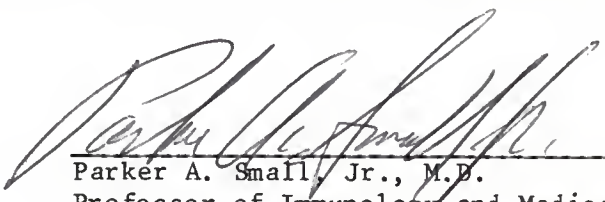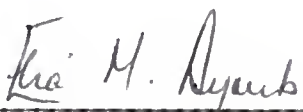University of Lund in Lund, Sweden.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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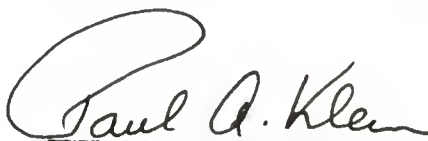Professor and Chairman of Immunology
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